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Visualization of the Specific Gene Transcription in the Nucleus with a Novel *In Situ* RNase Protection Method

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The ordinary *in situ* hybridization technique, using the labeled DNA or RNA probes, visualizes the mRNA species that have previously been transcribed and accumulated in the cytoplasm. In contrast, we aimed to visualize the transcription of particular genes itself by use of the hybridization between radioisotope-labeled newly transcribed cellular RNA and unlabeled RNA probes (riboprobes) followed by digestion of unhybridized RNA with RNase. In the present study, the cultured mouse 3T3-L1 cell line with or without the induction for the adipocyte was labeled with ^{3}H-uridine, fixed and was hybridized *in situ* with the complementary (antisense) or homologous (sense) riboprobe for 28S rRNA or glycerol-3-phosphate dehydrogenase (GPDH) mRNA, an adipocyte marker. Following extensive digestion with RNase, the signal for remaining radioactivity in nuclei was quantitatively analyzed with autoradiography. In the uninduced cells hybridized with 28S rRNA probes, a significantly stronger signal was obtained with the complementary probe than with the homologous probe. With respect to GPDH probes, the adipocyte-induced cells showed a significantly stronger signal with the complementary probe than with the homologous probe, whereas the uninduced cells showed no significant difference in signal with both probes. These results demonstrated the possibility of visualizing the transcription of particular genes in particular cells at particular time point by applying "*in situ* RNase protection" to histological specimens.

Key words: Transcription, *In situ* hybridization, RNase protection, Adipocyte differentiation

I. Introduction

In situ hybridization is a powerful tool in visualizing the expression of tissue- and cell-specific mRNA species in histological sections or cell culture systems. In the ordinary *in situ* hybridization technique, the DNA or RNA probe complementary in sequence to particular mRNA is labeled with radioisotope (RI) or non-RI markers and used for hybridization with fixed cells and tissues. The intensity of the hybridization signal, therefore, represents the net amount of particular mRNA species that has accumulated in the cytoplasm, and not necessarily the amount of the mRNA currently being transcribed. In the present study, we attempted to develop a novel histochemical technique based on labeling of the cellular mRNA rather than the probes. In this method, newly transcribed cellular RNA are labeled by an incorporation of RI-labeled ribonucleoside. Subsequently, the cells are hybridized *in situ* with an unlabeled RNA probe (riboprobe), followed by an extensive digestion of unhybridized RNA with RNase. The remaining signal for cellular radioactivity is expected to represent the particular gene transcripts that have been hybridized with the riboprobe and protected from RNase digestion. Subsequently, the autoradiography can quantitatively visualize the signal intensity. We tentatively call this method "*In situ* RNase protection".

A mouse cell line 3T3-L1 is known as an adipocyte precursor [5]. It can be induced to differentiate into the adipocyte by exogenous signals, typically by the culture medium containing insulin, a glucocorticoid and a cAMP-phosphodiesterase inhibitor [14, 15]. In the course of the differentiation, a number of specific marker proteins are expressed [reviewed in 10], including glycerol-3-phosphate dehydrogenase (GPDH, EC1.1.1.8) [4, 17, 18]. GPDH is a NAD-dependent cytosolic enzyme that plays a crucial role in the adipocyte metabolism by generating glycerol 3-phosphate, the major substrate for fatty acid acylation in triglyceride biosynthesis [16]. GPDH is estimated to

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occupy 2.7% of the total soluble protein in the adipocyte-induced cells [17].

In the present study, to validate *in situ* RNase protection, we first applied the method to detecting the transcription of 28S rRNA, which is one of the most abundant RNA species in mammalian cells and can be used as a marker of intact transcripts in tissue specimens [3, 20], in the undifferentiated 3T3-L1 cells. Subsequently, we applied the method to detecting the transcription of GPDH mRNA in the cells induced for adipocyte differentiation.

II. Materials and Methods

Cell culture

3T3-L1 cells were purchased from Human Science Resourses Bank, Osaka, Japan. They were cultured in Dulbecco's modified minimum essential medium (MEM; Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS; Life Technologies, Inc., Rockville, MD) at 37°C in an atmosphere of 95% air and 5% CO_2 and were subcultured by splitting 1 : 4 every 3 days. For the induction of adipocyte differentiation, subconfluent cells were incubated for 48 hr with MDI medium, which contained 0.5 mM methylisobutylxanthine (Nacalai Tesque, Kyoto, Japan), 10 μg/ml insulin (Sigma, St. Louis, MO) and 1 μM dexamethasone (Nacalai Tesque) in MEM plus 10% FBS, according to previous reports [14, 15]. Subsequently, MDI medium was removed and the cells were further incubated for 4 days in fresh normal medium (MEM plus 10% FBS) before they were subjected to RNA extraction or fixation. The subconfluent cells without MDI treatment were used as contrtol (uninduced) cells. To confirm the cytoplasmic lipid droplets induced in the adipocyte differentiation, the cells were examined under phase-contrast inverted microscopy and were fixed in 4% paraformaldehyde/0.1 M sodium phosphate buffer (pH 7.2) and stained with oil red.

Preparation of probes

Total RNA was isolated from the liver homogenate of *ddY* strain mice with a guanidinine-thiocyanate method [2], using a commercial kit (RNAzol™; Tel-Test, Friendswood, TX). The first strand cDNA was synthesized from 2 μg of the total RNA using Moloney murine leukemia virus reverse transcriptase (Toyobo, Osaka, Japan). According to the published cDNA sequences of mouse 28S rRNA (GenBank access number, X00525) [8] and mouse GPDH (J02655) [4], the following oligonucleotide primers were designed and purchased from Japan Bio-Service, Asagiri, Japan: rRNA 5′ primer, 5′-CTTGAAA-CACGGACCAAGGA-3′; rRNA 3′ primer, 5′-GTTCTGCTT-ACCAAAAGTGG-3′; GPDH 5′ primer, 5′-CAGACAC-CCAACTTTCGCAT-3′; GPDH 3′ primer, 5′-TGTTCCG-GGTGGTTCTGCAG-3′. Using these primers and Taq DNA polymerase (ExTaq; Takara Biomedicals, Kusatsu, Japan), a 546 bp fragment of 28S rRNA cDNA and a 500 bp fragment of GPDH cDNA were amplified from the reverse-transcription product by polymerase chain reaction in a DNA thermal cycler (MJ Research, Watertown, MA). They were then subcloned into the SmaI site of plasmid pGEM3Zf(+) (Promega, Madison, WI) and were confirmed of the identities by DNA sequencing. An aliquot of GPDH cDNA fragment was labeled with [α-^{32}P] dCTP (Dupont, Wilmington, DE) to specific activities of $\sim 10^9$ dpm/μg using a Megaprime DNA labeling system (Amersham Pharmacia Biotech, Uppsala, Sweden) and was used as probe for Northern hybridization. To generate the complementary (antisense) and homologous (sense) riboprobes for ISH, the plasmids containing 28S rRNA and GPDH cDNA inserts were linearized with Hind III or EcoRI (Toyobo) and transcribed *in vitro* with T7 polymerase or SP6 polymerase (Toyobo), respectively, in the presence of digoxigenin-conjugated UTP (Roche Diagnostics, Mannheim, Germany). Digoxigenin-labeled probes are widely used in the non-RI *in situ* hybridization [9], and we employed them to confirm the validity of RNA-RNA hybridization prerequisite for *in situ* RNase protection. The amount and the labeling efficiency of the products were estimated by electrophoresing them on an agaraose gel together with a standard RNA sample and by spotting them on a nylon membrane to detect digoxigenin immunoreactivity, respectively.

Northern blotting

The cells were cultured on 10 cm petri dishes with or without the treatment with MDI medium for the adipocyte induction. They were then solubilized in a guanidinine-containing solution (RNAzol™, Tel-Test) and the total RNA was extracted as described above. For Northern blotting, 20 μg aliquots of the total RNA samples were denatured by glyoxal, electrophoresed on 1% agarose gel and transferred to a nylon membrane according to Thomas [19]. The membrane was hybridized with ^{32}P-labeled GPDH cDNA and exposed for autoradiography.

In situ ***RNase protection***

The cells were cultured on 24 mm × 24 mm coverslips placed in 35 mm petri dishes, with or without the treatment for adipocyte induction as described above. In the last 1 hr of the culture period, the cells were incubated with the normal medium containing 20 μCi/ml of [5,6-^{3}H] uridine (1.9 TBq/mmol; Dupont, Wilmington, DE) to label the newly transcribed RNA. Subsequently, the cells were briefly rinsed in PBS and fixed with 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.2) for 30 min at room temperature. They were then washed twice with PBS containing 2 mg/ml of glycine for 15 min each, dehydrated with 70% ethanol for 5 min and with 100% ethanol for 30 min. Having air-dried, the coverslips were mounted with the cell side up on glass slides using nail-polish and were dried at 4°C for 3 days.

The ^{3}H-labeled cells with or without the adipocyte induction were hybridized *in situ* with the digoxigenin-

labeled complementary or homologous riboprobe for GPDH or 28S rRNA. The cell specimens on the glass slides were first dipped in 0.3% Triton X-100 in PBS for 30 min, followed by washing with PBS. They were then incubated with 2 μg/ml proteinase K in TE buffer (10 mM Tris-HCl/1 mM EDTA, pH 8.0) for 10 min at 37°C, fixed again in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) for 15 min and were washed in PBS containing 2 mg/ml glycine. They were then treated with 0.2 N HCl for 20 min, acetylated with 0.25% acetic anhydride/0.1 M triethanolamine (pH 8.0) for 10 min, and were prehybridized in a mixture composed of 50% deionized formamide, 2 × SSC, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.25% SDS, 1 × Denhardt's solution and 200 μg/ml tRNA for 2 hr at room temperature. After dehydration with a graded ethanol series, the cells were hybridized with the digoxigenin-labeled riboprobes at the concentration of approximately 20 μg/ml in the hybridization mixture (10% dextran plus prehybridization mixture). After incubation for 16–24 hr at 50°C, the cells were briefly rinsed with two changes of 2 × SSC and washed in 50% formamide/2 × SSC for 30 min at 50°C. They were then washed in 2 × SSC, rinsed briefly in TNE buffer (10 mM Tris-HCl, pH 8.0, 0.5 M NaCl, 1 mM EDTA) and were incubated with 50 μg/ml of RNase A (Roche Diagnostics) and 20 units/ml of RNase TI (Roche Diagnostics) in TNE buffer at 37°C for 1 hr. In most experiments, the cells were further digested with 100 μg/ml of DNase I (Sigma) in 50 mM Tris-HCl (pH 7.5) plus 10 mM $MgCl_2$ for 30 min at 37°C prior to RNase treatment. Subsequently, the cells were washed by two 10 min-washes in 2 × SSC and two 20 min-washes in 0.2 × SSC at 50°C.

For the detection of hybridized digoxigenin-labeled probes, some cell specimens were incubated with 2% (w/v) skimmed milk in PBS for 30 min at room temperature, rinsed briefly with buffer A (100 mM Tris-HCl, pH 7.5, 150 mM NaCl) and then incubated 4 hr at room temperature with alkaline-phosphatase-conjugated sheep anti-digoxigenin antibody (Roche Diagnostics) at 1 : 500 dilution in buffer A. After two 10 min-washes in buffer A and a brief rinse in buffer B (100 mM Tris-HCl pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$), the immunoreaction was visualized by incubating the cells with a BCIP/NBT substrate system (Dakopatts, Glostrup, Denmark) containing 5-bromo-4-chloro-3-indolyl-phosphate and nitroblue tetrazolium chloride for a few hours.

Other cell specimens were subjected to autoradiography for detection of the radioactivity representing RNase-protected transcripts. After dehydration in graded ethanol series supplemented with 0.3 M ammonium acetate, the cells were dipped in Kodak NTB-2 emulsion, dried and were exposed for 2 weeks in dark at 4°C. Following the development, the cells were stained with hematoxylin/eosine and subjected to observation with an Olympus BX50 microscope connected to a CCD video camera (Olympus/Victor KY-F55MD). In randomly selected fields, a total of approximately 1000 cells were counted in each specimen for the number of nuclear silver grains, and the mean number of grains per nucleus was compared between the specimens hybridized with complementary and homologous probes for 28S rRNA or GPDH. The statistical difference between two values was examined by Student's t test, and the difference with $p < 0.01$ was considered significant. All experimetns were repeated at least three times.

III. Results

Detection of 28S rRNA transcription

To establish the procedures of *in situ* RNase protection, we first applied this method to detecting the transcription of 28S rRNA in the uninduced cells. The conditions for the pretreatment and hybridization of cell specimens were essentially the same as those for ordinary *in situ* hybridization, except that RNase T1 was used together with RNase A in the washing procedure to facilitate digestion of unhybridized RNA. When the cells were subjected to autoradiography without preceding hybridization or washing procedure, abundant silver grains were obtained, with a much larger number in the nuclei (more than 50 per nucleus) than in the cytoplasm (Fig. 1A). These grains were considered to represent primarily the RNA that had been transcribed within the 1 hr-labeling period. By applying RNase digestion, the grains were substantially reduced in number in most cells, whereas about 20% of the cells remained to exhibit considerable numbers of grains in nuclei (Fig. 1B). These grains, however, were substantially reduced when the cells were digested with DNase prior to or after RNAse digestion (Fig. 1C). Therefore, in the subsequent experiments we included DNase digestion in the routine washing procedure for *in situ* RNase protection.

After *in situ* RNase protection with the complementary and homologous probes for 28S rRNA, some cell specimens were subjected to alkaline-phosphatase histochemistry to visualize the hybridization reaction (Fig. 2A, B). All the cells hybridized with the complementary probe displayed positive staining for 28S rRNA transcripts, which were localized in the cytoplasm and also in the nucleoli. In contrast, the homologous probe brought about no staining, confirming that the present complementary probe specifically hybridizes with 28S rRNA transcripts. Other cell specimens were analyzed with autoradiography (Fig. 3A, B). The silver grains representing the radioactivity that had escaped from RNase digestion were located primarily in the nuclei and occasionally in the cytoplasm. In a representative experiment, the average number of grains per nucleus obtained with the complementary probe was 3.08 ± 2.33 (mean $\pm$SD), which was significantly higher than 1.69 ± 1.40 obtained with the homologous probe ($p < 0.005$) (Fig. 3C). Three separate experiments brought about similar results. These results suggested that the complementary probe hybridized with the new transcripts of 28S rRNA gene

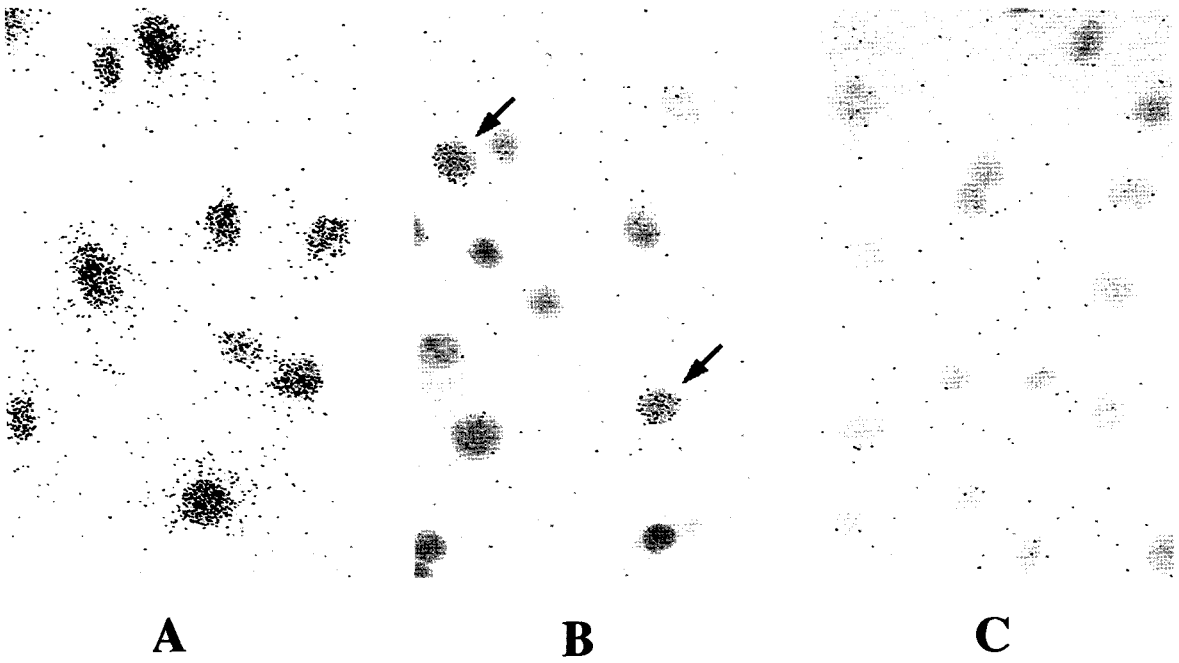

Fig. 1. Autoradiography of ^{3}H-labelled uninduced cells without hybridization. In the absence of RNase treatment (**A**), abundant silver grains are mostly located in the nucleus and partly in the cytoplasm. With RNase treatment (**B**), most cells have lost their grains, whereas a minor cell population (arrows) remains to have considerable numbers of grains. With DNase treatment prior to RNase treatment (**C**), all cells have lost their grains.

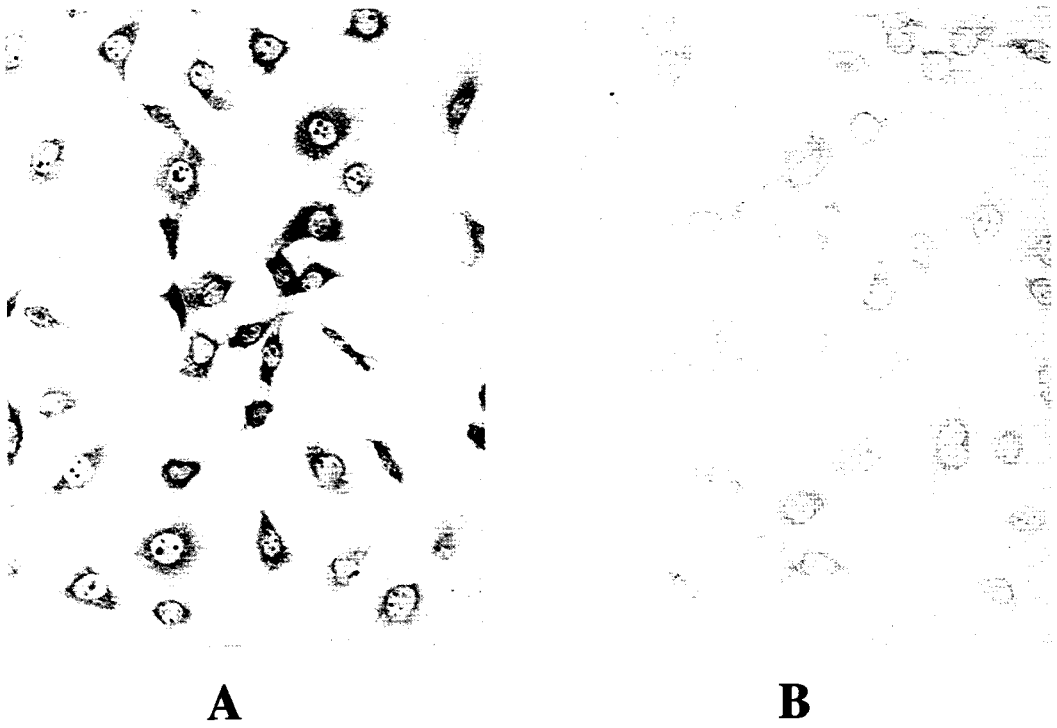

Fig. 2. Hybridization of the uninduced cells for 28S rRNA. With alkaline phosphatase histochemistry, a strong signal is demonstrated in the cytoplasm and nucleolus after reaction with digoxigenin-labeled complementary probe (**A**) but not with the homologous probe (**B**).

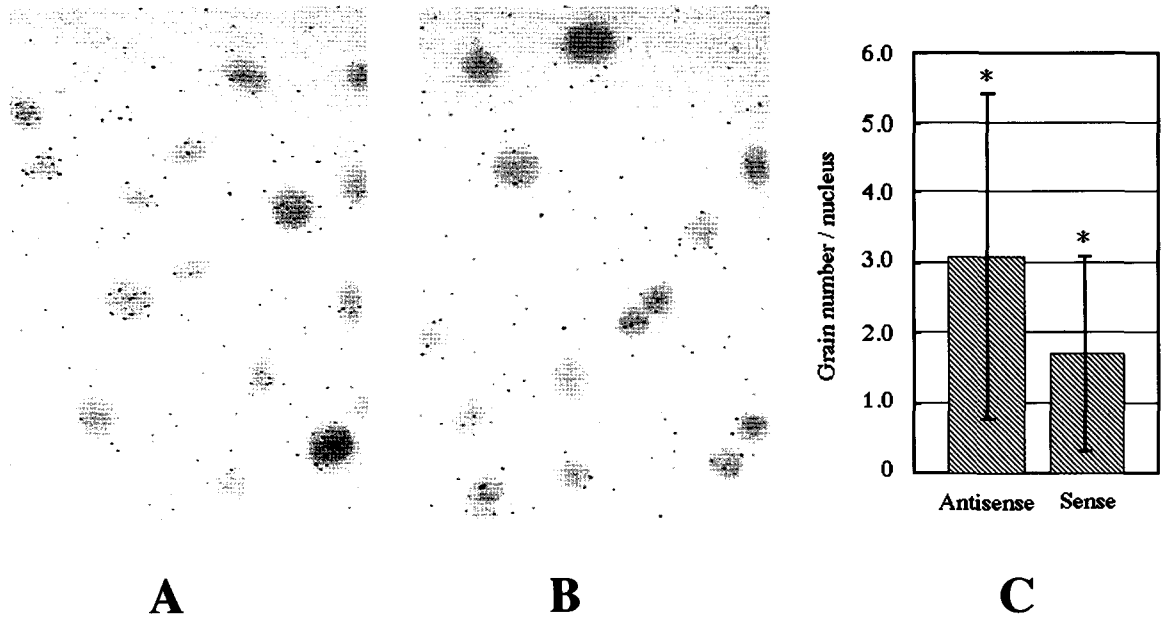

Fig. 3. *In situ* RNase protection of the uninduced cells for 28S rRNA transcripts. Autoradiography demonstrates silver grains in nuclei after reactions with the complementary (**A**) and homologous (**B**) probes. (**C**) Significantly larger number of nuclear grains is recognized in the cells hybridized with the complementary probe than in those with the homologous probe ($p < 0.005$; $n = 1011$ and 1017, respectively).

specifically protects them from RNase digestion.

Transformation of 3T3-L1 cells into adipocytes

After the induction for differentiaiton with MDI medium for 48 hr followed by a 4-day culture in normal medium, most cells had become round in shape and filled with cytoplasmic droplets as seen under the phase-contrast inverted microscope, indicating that they had transformed into adipocytes (Fig. 4A). The oil-red staining of the fixed cells further demonstrated neutral fat as the content of cytoplasmic droplets (not shown). In contrast, the control cells cultured to subconfluency without the induction with MDI medium remained to be fibroblast-like in shape and had no lipid droplets (Fig. 4B). North-

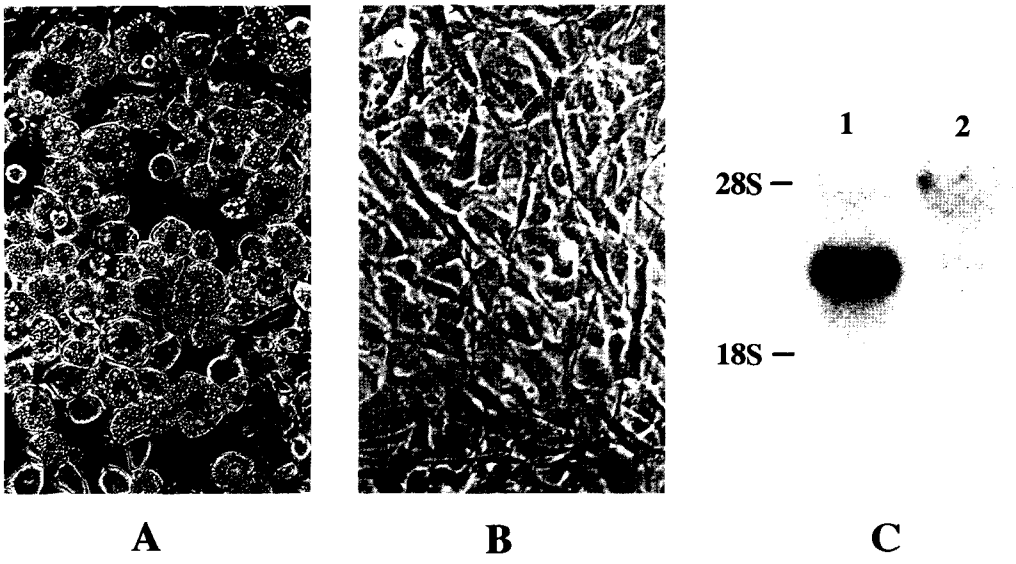

Fig. 4. Induction of 3T3-L1 cells into adipocytes. Phase-contrast microscopy showing the transformation of cells into adipocytes by treatment with MDI medium (**A**) but not with the normal medium (**B**). (**C**) Northern blot analysis of the total RNA demonstrates GPDH mRNA of ~3 kb length in adipocyte-induced cells (lane 1) but not in the uninduced cells (lane 2).

ern blot analysis exhibited an intense hybridization band ~3.0 kb in size corresponding to GPDH transcripts in the adipocyte-induced cells but not in the uninduced cells (Fig. 4C). These results indicated that in the present culture system MDI treatment effectively elicits the differentiation of 3T3 cells into adipocytes accompanied by a pronounced upregulation of GPDH.

Detection of GPDH transcription

The cells with or without the adipocyte induction were subjected to *in situ* RNase protection for GPDH in the same way as that for 28S rRNA. With alkaline-phosphatase histochemistry, the adipocyte-induced cells hybridized with GPDH complementary probe were intensely labeled in the cytoplasm, whereas those hybridized with the homologous probe were not, indicating that the present complementary probe hybridizes specifically with GPDH transcripts (Fig. 5A, B). Results of a representative experiment analyzing the autoradiography following *in situ* RNase protection are shown in Fig. 6A–C and Table 1. In the absence of either hybridization or washing procedure, the average number of nuclear silver grains was lower in the adipocyte-induced cells (31.0 ± 13.4) than in the uninduced cells (>50). After both procedures, although the silver grains were markedly reduced in number, the induced cells exhibited a significantly larger number of grains per nucleus with the complementary GPDH probe (1.09 ± 1.42) than with the homologous probe (0.51 ± 0.83) ($p < 0.005$). In contrast, the uninduced cells exhibited no significant difference between the grain numbers obtained with two probes. Three separate experiments brought about similar results. These results indicated that the complementary probe hybridized with the new transcripts of GPDH gene specifically protects them from RNase digestion.

IV. Discussion

In the present study, histochemical detection of digoxigenin-labeled riboprobes demonstrates the hybridization signal for 28S rRNA localized in both the cytoplasm and nucleolus of uninduced cells, and that for GPDH mRNA localized primarily in the cytoplasm of induced cells. These observations are consistent with the ability of *in situ* hybridization to detect the steady state of particular gene transcripts, i.e., the net result of their transcription, translocation from the nucleus to cytoplasm and partial degradation in both the nucleus and cytoplasm. In contrast, the radioactivity obtained by 1-hr incorporation of ^{3}H-uridine is mostly localized to the nucleus and is readily eliminated by RNase, suggesting that it represents the new transcripts. The presence of the minor population of uninduced cells exhibiting the grains highly resistant to RNase digestion is difficult to interpret. Since these grains are substantially reduced in number with DNase treatment, a likely explanation is that they represent the partial incorporation of ^{3}H-uridine into the replicating DNA of S-phase cells through intracellular metabolism. Alternatively, it is also possible that some newly tran-

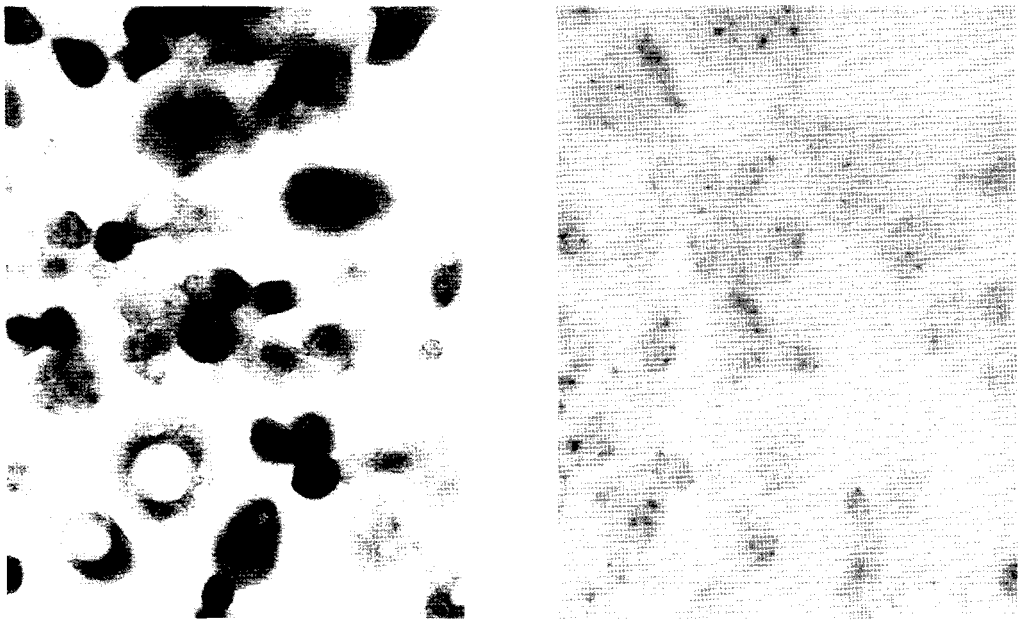

Fig. 5. Hybridization of adipocyte-induced cells for GPDH mRNA. With alkaline phosphatase histochemistry, a strong signal is demonstrated primarily in the cytoplasm after hybridization with digoxigenin-labeled complementary riboprobe (**A**) but not with the homologous probe (**B**).

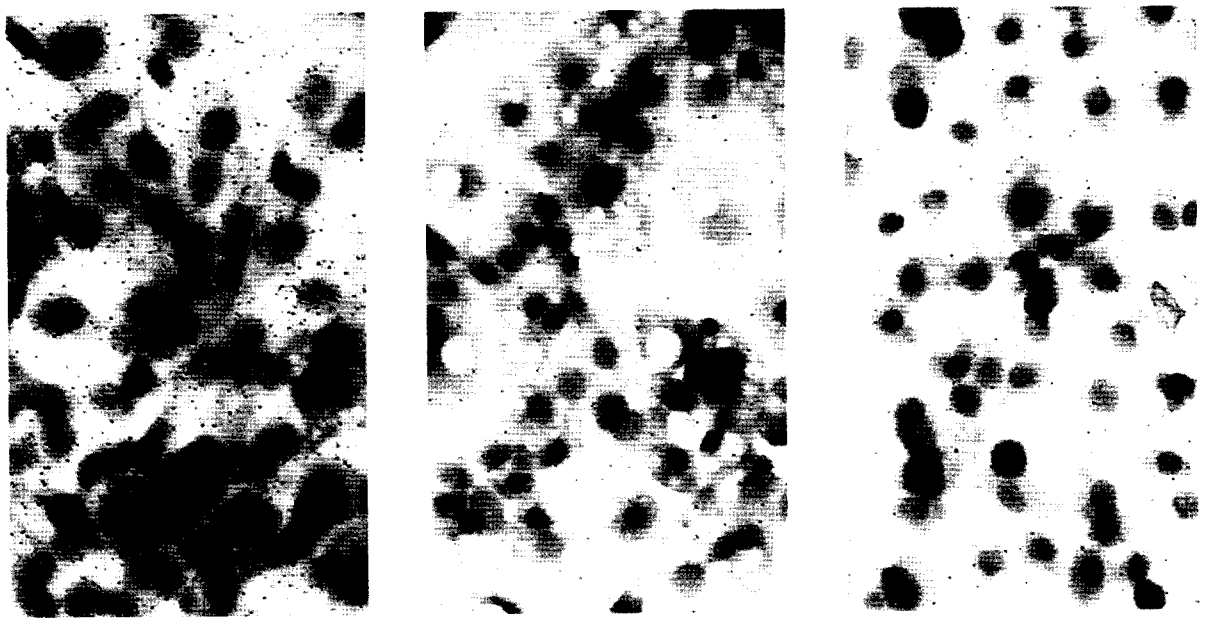

Fig. 6. *In situ* RNase protection of adipocyte-induced cells for GPDH transcripts as shown with autoradiography. In the absence of either hybridization or RNase treatment (**A**), abundant silver grains are present in nuclei. After RNase treatment, larger numbers of nuclear grains are recognized in the cells hybridized with the complementary probe (**B**) than in those with the homologous probe (**C**).

Table 1. In situ *RNAse protection assay of GPDH transcription in 3T3-L1 cells induced or uninduced for adipocyte differentiation.*

Adipocyte induction	Probe	RNAse digestion	Grain number/nucleus (mean ± SD)	Cell number counted
+	–	–	31.0 ± 13.4	201
	complementary	+	1.09 ± 1.42*	1007
	homologous	+	0.51 ± 0.83*	1001
–	–	–	> 50.0	212
	complementary	+	1.28 ± 1.38	1005
	homologous	+	1.36 ± 1.42	1010

* $P < 0.005$

scribed RNA are non-specifically protected from RNase digestion through binding with DNA under some cellular conditions. The combination of RNase and DNase therefore appears to be indispensable in the washing procedure. The present results demonstrate that the new transcripts of both 28S rRNA gene and GPDH gene are specifically protected from RNase degestion by the hybridization with respective complementary riboprobes.

Out of the total new transcripts in the mammalian cell nucleus, it is estimated that 39% are the rRNA precursor and 58% are heterogenous nuclear RNA (hnRNA), a mixture of mRNA precursors of various sizes [1]. The rRNA precursor, 45S in size, is then processed into 18S, 28S and 5.8S rRNA, which start to leave the nucleus as ribosomal subunits in about half an hour after the transcription [13]. In contrast, hnRNA are subjected to a progressive degradation in the course of their splicing to form mRNA, which start to leave the nucleus in about half an hour after the transcription [12]. As a result, the mature mRNA in cytoplasm account for less than 5% of the steady-state cellular RNA [1]. In the present study, a majority of the silver grains obtained with 1-hr incorporation of ^{3}H-uridine is located in the nucleus, suggesting that the labeled transcripts consist mostly of the rRNA precursor and hnRNA and partly of the mature rRNA, tRNA and mRNA.

In the *in vitro* system, RNase protection assay, based on the principle that double-stranded RNA are more resistant to RNase than are single-stranded RNA, is commonly used to detect particular mRNA species by

hybridizing them on filters with labeled riboprobes followed by RNase digestion [11, 21]. Also, in the ordinary *in situ* hybridization with riboprobes, this principle is used in the washing of unhybridized riboprobes with RNase. In both cases, however, the probe side is labeled. On the other hand, there is a technique called nuclear run-on assay, in which the newly transcribed RNA in the nucleus are labeled and subjected to hybridization on filters with unlabeled probes to detect the transcription of particular genes [6, 7]. *In situ* RNase protection developed in the present study is regarded as a technique that has combined RNase protection assay and nuclear run-on assay and has applied it to the *in situ* system. The results indicate that this method can detect both the precursors of 28S rRNA and of GPDH mRNA that are currently being transcribed.

In the present results, since the number of nuclear silver grains in the uninduced cells without RNase digestion is more than 50 per nucleus, at least 20 grains (39%) are considered to represent the rRNA precursor. As the number of grains remaining after RNase digestion is 3.08 when the cells are hybridized with complementary 28S rRNA probe and 1.69 with the homologous probe, only about 1.39 grains are specifically protected from RNase digestion. Although this corresponds to less than 7% of the presumptive amount of the rRNA precursor, it may be reasonable considering that 1) only the portion of the rRNA precursor that has hybridized with 546 base-long 28S rRNA probe is expected to be protected; 2) the efficiency of hybridization between the probe and rRNA precursor may not be high enough; and 3) the resistance of the hybridized rRNA precursor to RNase digestion may not be perfect. In a similar consideration, out of 31 silver grains per nucleus in the adipocyte-induced cells without RNase digestion, 18 grains (58%) or less may represent hnRNA, of which only a portion may be occupied with GPDH mRNA precursors. As the number of grains remaining after RNase digestion is 1.09 when the cells are hybridized with complementary GPDH probe and 0.51 with the homologous probe, only about 0.58 grains, corresponding to ~3% of the presumptive amount of hnRNA, are specifically protected from RNase digestion. This value, however disappointing it is, may represent the maximum sensitivity of detecting the transcription of any abundantly-expressed genes using the present experimental protocol.

It is notable that the number of silver grains obtained without RNase digestion is larger in the uninduced cells than in the induced cells, presumably because of the higher overall transcriptional activity of the former. Moreover, the number of grains obtained by the hybridization with homologous GPDH probe followed by RNase digestion is also larger in the uninduced cells than in the induced cells. This result suggests that these grains are produced not only by the pure background of autoradiograms but also by a certain fraction of transcripts that is refractory to RNase digestion even without protection. If the amount of the total transcripts is larger, that of the refractory transcripts should also be larger. This means that the absolute number of grains cannot be compared between the different cell populations, and thus the evalulation of *in situ* RNase protection is possible only by comparing the values obtained with the complementary and homologous probes in the same cell population.

The present study has proposed the possibility of visualizing the specific gene transcription in the nucleus using novel *in situ* RNase protection. This method is potentially useful in analyzing regulation in the expression of particular genes in particular cells in shorter time periods, and will display its ability to the full when applied to histological sections. However, for the present this method appears to be far from practical use because of its low sensitivity. The possible ways of increasing the sensitivity of *in situ* RNase protection are 1) to raise the intensity of initial labeling of transcripts; 2) to use multiple riboprobes for protecting a larger compass of the transcript sequence; and 3) to adjust the conditions of hybridization and/or RNase digestion for producing the maximum signal/background ratio. These should be examined in the future studies.

V. Acknowledgments

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