Labeling of RNA Transcripts of Eukaryotic Cells in Culture with BrUTP Using a Liposome Transfection Reagent (DOTAP®)

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ABSTRACT

Bromouridine-triphosphate (BrUTP), when introduced into eukaryotic cells in culture, substitutes for UTP during transcription, thereby labeling pre-mRNA for detection by immunochemical methods. In earlier studies, BrUTP was uptake by means of microinjection or by exposing isolated nuclei or permeable cells to BrUTP. We describe here a simple method for the uptake of BrUTP into monolayers of growing cells using a cationic liposome transfectant (DOTAP®). Within minutes, DOTAP mediates uptake of BrUTP both at 37°C and 4°C. This is followed by incorporation into RNA in the nucleus upon further incubation under culture conditions. In this way, large numbers of actively growing cells may be subjected to biochemical experiments.

INTRODUCTION

Various liposome formulations have been used to transfect plasmid DNA into eukaryotic cells in culture. Among such lipofectants, the synthetic cationic amphiphile DOTAP® (N,N,N,N′-tetraakis(2,3-dioleoyloxy)propyl)-N,N,N,N-trimethylammonium methylsulfate) (8) has been shown to mediate uptake of plasmid also in the presence of a culture medium containing serum. It is metabolized in the cells and has proven to be less cytotoxic than other amphiphiles (6). DOTAP has also been used successfully for internalization of an 18-mer DNA oligonucleotide, and it was shown in these experiments to protect the oligonucleotide from degradation by nucleases in serum and cells (1). Recently, another cationic liposome (LipofectAMINE®) has been shown to mediate delivery of biochemical and biologically active proteins into cells (7).

In the study of the trafficking of the human immunodeficiency virus (HIV) type 1 regulatory protein Rev in cells in culture (4,5), it was found to be functionally important to distinguish between nucleoplasmic sites of transcription/splicing and separate sites containing a high concentration of RNA-processing factors (speckles) apparently not actively involved in RNA splicing (2,3,9,11). Since distinction between different morphological and functional compartments of the nucleoplasm is highly relevant for the understanding of gene expression, and since the subcellular distribution of RNA-processing factors is sensitive to variation of fixation protocols (4), direct visualization of nascent RNA labeled in situ provides an important landmark.

To localize transcription sites in the nucleus, we microinjected the uridine triphosphate (UTP) analogue 5-bromo-uridine 5′-triphosphate (BrUTP) into a HeLa cell line that had been stably transfected with the rev gene. Under these conditions, BrUTP was previously shown to substitute for UTP in nascent RNA (3,9); however, it could interfere with further processing of RNA (10). Various permeabilization procedures have also been used to internalize BrUTP, since BrUTP is not taken up by intact cells (3,9). Usually, only a limited number of BrUTP-labeled cells can be obtained by microinjection, and permeabilization may adversely affect the subcellular antigen distribution to which Rev distribution should be related (4).

We therefore examined the possibility of using the DOTAP transfection reagent as a vector for the uptake of BrUTP into growing HeLa and COS-1 cells. Large amounts of cells produced brominated RNA by this procedure, which may prove useful for various biochemical studies.

MATERIALS AND METHODS

Cell Cultures

A HeLa cell line, which was constitutively transfected with the HIV-1 regulatory gene rev (HeLa/crev8; a gift from Dr. Bryan Cullen, Howard Hughes Medical Institute, Durham, NC), and COS-1 cells were cultured on 12-mm-diameter coverslips. In the BrUTP-labeling experiments, the cells were grown at 37°C under 5% CO₂ in Dulbecco’s modified Eagle medium (Whittaker, Vervies, Belgium) supplemented with gentamicin and 5% or 10% fetal calf serum (Advanced Protein Products, Brockmoor, West Midlands, UK). The HeLa/crev8 cell line was otherwise propagated in Iscove’s modified Dulbecco’s medium (Whittaker) with 0.5 mM methotrexate. The cells were grown to 60% to 80% monolayer density. Alternatively, a lesion was made in a confluent monolayer by scratching, followed by further cultivation at 37°C in order to have actively dividing cells along the lesion border.

Preparation of DOTAP/BrUTP Complexes and Application to Cell Culture

DOTAP (Boehringer Mannheim, Mannheim, Germany) and BrUTP (Sigma Chemical, St. Louis, MO, USA) solutions were prepared in 20 mM HEPES buffer in saline, pH 7.4, according to the manufacturer’s directions for transfection of plasmids with DOTAP. After complexing BrUTP and DOTAP for 10 min at room temperature, 150-µL aliquots containing 0.1, 0.5, 2.5 and 10 mM of BrUTP were added to 16-mm wells with cells on coverslips and left to preincubate for 15 min at 37°C under 5% CO₂. Preincubation at room temperature and at 4°C was also attempted. After two washes with phosphate-buffered saline (PBS), pH 7.4, some coverslips were removed for immunostaining. The remaining coverslips were incubated further with culture medium containing 10% fetal calf serum at 37°C for periods of 5 min and up to 18 h (chasing) before processing for immunofluorescence microscopy. To inhibit transcription by DNA-dependent RNA polymerases (RP) I, II and III, 0.04, 5 and 100 µg/mL, respectively, of actinomycin D (Cosmegen®; Merck Sharp & Dohme B.V., Haarlem, The Netherlands) were added to parallel cultures during preincubation and chasing. Following permeabilization in cold absolute methanol for 3 min, coverslips with cells were also treated with 50 µg/mL RNase A (DNase-free).
(Qiagen, Chatsworth, CA, USA) at room temperature for 30 min.

**Microinjection**

Cells on coverslips were injected into the cytoplasm using a micromanipulator (Model 5171; Eppendorf, Hamburg, Germany) and glass micropipet (Femtotips®; Eppendorf). The injection buffer contained 100 mM BrUTP in 140 mM KCl and 2 mM (piperazine-N,N′-bis[2-ethane-sulfonic acid]). (PIPES) pH 7.4 (9). In control experiments, 100 µg/mL actinomycin D were included in the injection buffer. After injection, the cells were cultivated for 1 h before processing for immunofluorescence analysis.

**Immunofluorescence Analysis**

Coverslips with cells were washed twice in PBS and fixed in freshly prepared 3.7% paraformaldehyde for 20 min at room temperature. After washes with glycine-PBS and PBS, the cells were treated with absolute methanol at -20°C for at least 1 h. The immunostaining was performed according to Wansink et al. (9) and included overnight incubation at 4°C with a monoclonal rat antibody against BrdU (Sera-Lab, Crawley Down, England, UK), followed by biotin-conjugated donkey anti-rat IgG (H+L; Jackson Immuno-Research Laboratories, West Grove, PA, USA) and streptavidin-FITC conjugate (Amersham International, Little Chalfont, Bucks, England, UK). Antibodies against BrdU have been shown also to react strongly with BrU. The coverslips were mounted on slides in SlowFade™ reagent (Molecular Probes, Eugene, OR, USA) and viewed by ordinary immunofluorescence microscopy or scanned by confocal laser microscopy using a Z resolution of 0.9 or 1.2 µm.

![Figure 1. BrUTP labeling of RNA after exposure of HeLa cells to BrUTP-DOTAP complexes.](image)

Cells were preincubated with 1 mM BrUTP complexed with DOTAP and chased for 1 h by incubation with culture medium containing 10% fetal calf serum (A–C). D) 5 µg/mL of actinomycin D were added during preincubation and chasing (photo is intentionally overexposed to visualize the borders of nonlabeled nuclei). Bars: A = 6 µm; B–D = 2.5 µm.
RESULTS

Pilot experiments indicated that BrUTP-DOTAP complexes were rapidly taken up by the cells. After preincubation of cells with BrUTP-DOTAP for 5, 10 and 15 min at 37°C, BrUTP was detected in the cytoplasm and nucleoplasm of most cells, somewhat less after 5 min than after 10 and 15 min. By applying the higher concentration of BrUTP (10 mM), some cells showed the intense nuclear immunostaining characteristic of brominated RNA (see below). BrUTP was also taken up at about the same rate after preincubation at room temperature and at 4°C. Following washing of the cells with PBS and further incubation (chasing) at 37°C with culture medium containing serum, intense nuclear immunostaining appeared, in addition to weak cytoplasmic staining. A preincubation period of 15 min at 37°C was used in further studies. Identical immunostaining patterns were obtained using the two cell lines HeLa/crev8 and COS-1.

Localization of BrUTP in Growing Cells

The intense nucleoplasmic immunostaining seen after chasing appeared, at higher magnification, to be caused by evenly distributed granules. The nucleoli remained unstained, and the cytoplasm stained weakly and variably (Figure 1, A–C). When preincubation and chase experiments were run in the presence of 5 or 100 µg/mL actinomycin D, the intense nuclear immunostaining did not appear (Figure 1, D). By using a low concentration of actinomycin D (0.04 µg/mL), which inhibits RPI, no decrease in nuclear immunostaining was observed. Confocal laser microscopy of the preparations with Z scanning showed mostly homogeneous granular immunostaining throughout the nucleoplasm and “empty” nucleoli.

When BrUTP-DOTAP was added to confluent monolayers, only scattered cells with nuclear immunostaining were seen. When a lesion was scratched in the monolayer, rapidly growing cells along the border of the lesion incorporated BrUTP in their nuclei (Figure 2, A and B) at a higher rate.

Dose of BrUTP, and Preincubation and Chase Dependency

Cells on coverslips were exposed to BrUTP complexed with DOTAP at nucleotide concentrations ranging from 0.1 to 10 mM. Only a few scattered cells became immunostained (intense nuclear staining) when exposed to 0.1 mM BrUTP and chased for 1 h, while up to 40% of the cells became intensely stained when 10 mM were applied. Exposure of the cells to BrUTP at concentrations of 1 or 2.5 mM and a chase of 15 min resulted in distinct RNA labeling of 20%–30% of the cells. Increasing numbers of labeled cells were seen after a prolonged chase of up to 2 h (Figure 3, A–D). The signal strength also increased correspondingly, although it varied somewhat among cells. After 3 h, the nuclear signal had become weaker, but more cytoplasmic staining was seen; and after 18 h, only weak nuclear staining was detectable in addition to cytoplasmic staining. Also, when preincubation was performed at 4°C, significant RNA labeling was seen after 4 min of chasing, which increased in strength, but with identical staining pattern, after 8, 12 and 16 min. RNase treatment removed all BrUTP nuclear labeling and most of the cytoplasmic labeling (some very fine granules were left) in cultures chased for up to 18 h.

The cytoplasmic staining appearing after prolonged chase consisted of coarse and irregular granules or patches.

Microinjection of BrUTP

The nuclear immunostaining pattern after microinjection of BrUTP and chasing for 1 h (Figure 4, A–C) was the same as that seen in the corresponding BrUTP-DOTAP experiments. In the cytoplasm, the staining pattern was somewhat coarser and partly microglobular (Figure 4C), resembling the pattern seen after prolonged chasing with the lipofectant (see Figure 3D).

DISCUSSION

The present results show that the cationic lipofectant DOTAP, in addition to functioning as a transfectant for plasmids (6,8), an oligonucleotide (1) and proteins (7), efficiently and rapidly mediates uptake of the halogenated base analogue BrUTP into eukaryotic cells in culture. The RNase experiments showed that BrUTP became incorporated into RNA and not into DNA. The protocol recommended by the manufacturer for transfection of plasmid DNA into eukaryotic cells includes preincubation of cells in suspension or monolayers with DOTAP-DNA com-
plexes for 3–24 h before washing and the addition of the culture medium with serum. During this preincubation period, considerable labeling of RNA transcripts takes place, and further time-related studies of transcriptional events cannot be performed. In the present experiments, preincubation of BrUTP-DOTAP complexes for 15 min at 37°C or in the cold appeared to be sufficient to internalize BrUTP in the two cell lines examined. Only scattered cells showed nuclear labeling during this period. After washing and further incubation (chasing) of the cells with culture medium at 37°C, increasing numbers of cells showed nuclear immunostaining, which increased in intensity with time. No staining appeared in the nucleolus at any time, which may be due to predominant RP II activity and/or a certain degree of inaccessibility of antibody to the nucleolus as discussed in References 3 and 9. The actinomycin D experiments show that the intense nuclear immunostaining is primarily caused by brominated RPII transcripts, as was shown earlier when microinject-

Figure 3. Immunostaining of HeLa cells after exposure to 1 mM BrUTP-DOTAP and chasing for increasing times. A) 15 min; B) 30 min; C) 1 h; D) 2 h. Bars (A–D): 25 µm.

Figure 4. HeLa cells microinjected with BrUTP. Cells were injected with BrUTP and incubated for 1 h with culture medium containing serum before immunostaining. Bars: A = 25 µm; B = 6 µm; C = 2.5 µm.
ed or permeable cells were examined (9). The weak staining that remained in the cytoplasm after actinomycin D treatment most likely represents nonincorporated BrUTP. The nature of the RNase-sensitive labeling seen in the cytoplasm was not studied further.

In addition to being more convenient than microinjection, the DOTAP method makes it possible to label an almost unlimited number of cells for further cytological and biochemical studies. Since BrUTP is sensitive to UV light, UV-cross-link experiments may prove useful to identify RNA-associated protein. The incorporation of BrUTP into RNA was shown to be dose- and time-dependent. Upon prolonged chasing, the nuclear label became weaker and more label appeared in the cytoplasm. After 18 h, widespread cell degeneration was seen with the higher concentrations of BrUTP. As expected, actively growing cells incorporated BrUTP in RNA at a higher rate, as was evident after scratching a lesion in a confluent monolayer.

Since BrUTP/DOTAP is taken up by the cells in culture within minutes at low temperature, transcription of RNA and possibly some post-transcriptional events may be studied in growing cells. However, transcription and pre-mRNA processing are completed very rapidly in the cell (discussed in more detail in References 3 and 9), and retardation of these processes may be necessary in order to identify actual transcription sites.

Internalization of BrUTP into eukaryotic cells in culture and incorporation into newly synthesized RNA can accordingly be achieved without having to resort to microinjection or permeabilization of the cells.

REFERENCES


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Highly Sensitive Method to Detect mRNAs in Individual Cells by Direct RT-PCR Using Tth DNA Polymerase

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ABSTRACT

A new method for detecting the expression of low-abundance mRNA molecules has been developed that combines the sensitivity of PCR, the high efficiency and specificity of reverse transcription (RT) using Tth DNA polymerase at high temperature, and the enhancement of sensitivity and specificity of nested PCR. This method is highly sensitive, reproducible and allows the detection of mRNAs in individual cells by direct RT-PCR.

INTRODUCTION

Reverse transcription polymerase chain reaction (RT-PCR) is the most sensitive technique available to detect low-abundance mRNA transcripts. Although several protocols using this technique have been reported to detect mRNA in single cells (3,5,6,8), they all require an RNA isolation step, which is time-consuming and is accompanied by an unavoidable loss of material, thereby making quantitation difficult. Furthermore, RNA extraction from single cells often requires expensive equipment not readily available in most laboratories. Recently, different reports explored the possibility of amplifying mRNA from single cells by direct RT-PCR to avoid some of these problems (1,2,4); however, a precise quantitative analysis of the limits of these methods was not provided. Moreover, these techniques were either time-consuming or needed radioactive or special material. Additionally, multistep manipulation of the reverse transcription product (1) was necessary or the Tth enzyme property enabling reverse transcription at high temperatures was not used (2,4). We report here a highly sensitive method to detect mRNAs in individual cells by direct RT-PCR using Tth DNA polymerase. This method circumvents the problems...