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In situ Localization of the Human Multidrug-resistance Gene mRNA Using Thymine-Thymine Dimerized Single-stranded cDNA

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In order to detect the mRNA transcribed from the multidrug-resistance gene (MDR1), thymine-thymine (T-T) dimerized single-stranded DNA probes have been utilized for hybridization with mRNA either on nitrocellulose filters or in cells and tissues. S1 nuclease digestion rather than sonication was used to obtain short T-T dimerized single-stranded DNA (300–400 bases) so that they could penetrate well into the cytoplasm. The hybridized T-T DNA was detected immunohistochemically using rabbit anti-T-T DNA antibody (Ab) and peroxidase-labeled goat anti-rabbit IgG Ab. Employing this system, MDR1 mRNA could be localized clearly in the human multidrug-resistant cell lines K562/ADM, CEM/VLB, 2780^{AD}, and KBC4 cells as well as in human fetal kidney and gastric carcinoma. Furthermore, our system successfully detected the expression of MDR1 mRNA in cell lines of increasing resistance. These results paralleled results obtained at the protein level by immunohistochemistry. The analysis of MDR1 RNA expression by this *in situ* hybridization technique should be useful in the study of normal human tissues and tumor samples expressing the MDR1 gene.

Key words: MDR1 gene — *In situ* hybridization — Thymine-thymine dimerized cDNA

It has been shown that several normal human tissues as well as some human cancers possess P-glycoprotein recognized by monoclonal antibodies (MAb).[1-5] To confirm that P-glycoprotein on the cell surface is a product of the cell on which it appears and to detect expression of the MDR 1 gene at a single cell level, it is important to localize mRNA which codes for P-glycoprotein.[6,7] Therefore, we have developed an *in situ* hybridization technique for the analysis of expression of the MDR1 gene in individual cells to determine which cell types in specific organs are expressing this gene and if possible to use as a sensitive diagnostic test for the expression of the MDR1 gene in human tumors. For this purpose, we have utilized an *in situ* hybridization technique using thymine-thymine (T-T) dimerized cDNA developed by Nakane *et al.*,[8] because this method allows the localization of mRNA specifically and reproducibly with high resolution, whereas in the case of *in situ* hybridization using radiolabeled cDNA one must be careful in interpreting results close to the low limit of resolution of this assay because of the background of grains.[7]

This paper describes the successful localization of MDR1 mRNA in fresh cells and tissues, by means of *in situ* hybridization analysis using non-radioactive single-stranded (ss) cDNA.

MATERIALS AND METHODS

Cell lines and tissues K-562 (human myelogenous leukemia) was established by Lozzio and Lozzio,[9] and K-562/ADM, an *in vitro*-induced adriamycin-resistant subline of K-562 was established by Tsuruo *et al.*[10] An ovarian cancer cell line, A2780, and its adriamycin-resistant strain, 2780^{AD}, were kindly supplied by Drs. R. F. Ozols and T. C. Hamilton, National Cancer Institute, USA.[11,12] KB-3-1, a drug-sensitive subclone of KB, and its colchicine-selected multidrug-resistant derivatives KB-8-5, KB-C1 and KB-C4 have been previously described.[7,13] CCRF-CEM, and its vinblastine-resistant lymphoma cell line, CEM/VLB were kindly supplied by Dr. W. T. Beck.[14-16] The cell lines were passaged twice weekly in RPMI 1640 with 10% FCS.

A gastric carcinoma termed Sc2 was supplied by Dr. Y. Sugimoto, Japanese Foundation for Cancer Research, Tokyo. Fetal tissues were provided by Dr. S. Itoyama, Saitama Medical Center, Saitama Medical School. The tissues were immediately snap-frozen in *n*-hexane.

DNA probes A 2.2 kb cDNA probe, comprising the first half of the human MDR1 cDNA, was excised from pMDR2000[17] with *Bam*HI and *Hin*dIII, and subcloned

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into the *Bam*HI-*Hin*dIII sites of M13mp19. The resultant phage M13mpM1 was purified by PEG/NaCl precipitation.[18] After the removal of host DNA and RNA by digestion with ribonuclease A and DNase I, the ss DNA containing the complementary sequence to MDR1 mRNA was extracted from phage M13mpM1 and used as a non-redioactive probe. M13mp19 was used as a negative control probe.

Antibodies Rabbit anti-T-T dimer antibody (Ab) was a gift from Dr. H. Yoshida, Kyoto University, School of Medicine.[19] The Fab fragment of goat anti-rabbit IgG Ab conjugated with horseradish peroxidase (HRP) (Sigma, type VI) was prepared according to the method of Wilson and Nakane[20] and was used as the second Ab. MRK 16 Ab reacting with P-glycoprotein was prepared by Hamada and Tsuruo.[21]

T-T dimerization of probe DNA T-T dimers were introduced into the probe DNAs by UV-irradiation as described in detail previously.[8] UV doses for probe DNAs were 3,000, 5,000, and 7,000 J/m^2 and the optimal dose was determined by the dot blot hybridization method.[8, 22]

S1 nuclease digestion of T-T dimerized DNA probes To shorten the length of T-T dimerized DNAs, the probe DNAs were treated with S1 nuclease instead of sonication.[19] Briefly, 18 μl of TE buffer containing the DNA probes (50 μg/ml) was prewarmed at 37°C for 15 min. Then, 2 μl of S1 nuclease buffer (2 *M* NaCl, 0.5 *M* sodium acetate, pH 4.5, 10 m*M* $ZnCl_2$, and 5% glycerol) was added. Thereafter, 2 μl of S1 nuclease buffer containing S1 nuclease (0.5 units/μl, Takara Shuzo Co., Kyoto) was added and the mixture was incubated at 37°C for 10 min. The mixture was then boiled for 10 min.

Determination of DNA size The probe DNAs were heated in a boiling water bath for 10 min, mixed with bromophenol blue-xylene cyanol, and applied to a 1% agarose gel. Electrophoresis was carried out at 40 V in an ice bath. As a size marker, *Alu*I digest of pBR322 DNA was used. After electrophoresis, the gel was stained with ethidium bromide and analyzed under UV illumination.

Isolation of cellular RNA from K-562/ADM, KB-3-1, KB-8-5 and KB-C1 cells Cellular RNA was isolated by the method of Maniatis *et al.*[23] The respective cell line was washed twice with phosphate-buffered saline (PBS), lysed with NP-40 lysis buffer (5 *M* NaCl, 1 *M* $MgCl_2$, 1 *M* Tris, pH 8.6, 0.5% NP-40, 10 m*M* vanadyl-ribonucleoside complexes), and gently mixed. The solution was centrifuged at 5,000 rpm at 4°C for 5 min. The pellet was suspended in a mixture of 0.1% SDS and 5 m*M* EDTA/PBS and chloroform/phenol (1:1) of equal volumes and the suspension was rotated at room temperature for 20 min, then centrifuged at 3,000 rpm at the same temperature for 10 min. The upper layer was mixed with equal volumes of chloroform/isoamyl alcohol (99:1), rotated for 20 min, and centrifuged at 3,000 rpm for 10 min, all at room temperature. The upper layer was mixed with 1/10 volume of 3 *M* sodium acetate and 2.5 volumes of ethanol, and was allowed to stand at −20°C overnight. Thereafter, the mixture was centrifuged at 5,000 rpm at 0°C for 10 min. The precipitate was dissolved in TE buffer (0.01 *M* Tris-HCl, pH 7.4, 0.1 m*M* EDTA).

Detection of T-T dimerized DNAs First, the detection sensitivity of the T-T dimerized DNAs was examined immunochemically by using an anti-T-T antibody. ss M13mpM1 and ss M13mp19 were irradiated at the rate of 5 J/m^2/s for 10 min (3000 J/m^2), 17 min (5000 J/m^2), or 23 min (7000 J/m^2), followed by S1 nuclease treatment. Ten pg to 10 ng of either the ss M13mpM1 or M13mp19 DNA fragments was backed on nitrocellulose membranes. The membranes were first reacted with 200 μl of 32 μg/ml anti-T-T IgG for 3 h and followed by reaction with 200 μl of 1 μl/ml of peroxidase-labeled goat anti-rabbit IgG for 3 h. The membranes were then histochemically stained for peroxidase using diaminobenzidine-4 HCl and H_2O_2 as substrates.

Dot blot hybridization Before *in situ* hybridization, dot blot hybridization was carried out to determine optimal cDNA probe concentrations. Various quantities (10 ng to 20 μg of RNA in 5 μl of TE buffer) of irradiated denatured RNA were dotted on strips of nitrocellulose filters. The filters were dried, heated at 80°C for 2 h, and prehybridized at 42°C for 2 h. Hybridization was performed at 43°C for 15 h as described elsewhere.[22]

***In situ* hybridization** About one hour prior to the start of the procedure, 5 μm sections of the tissues embedded in O.C.T. compound and frozen were made, placed on gelatin-coated glass slides[22] and air-dried. The cells were spotted on Denhardt's solution-coated slide glasses[24] using a Cytospin centrifuge (Shandon Instruments Inc., USA). The sections and cells were fixed with either ethanol-acetic acid (3:1) mixture or 4% paraformaldehyde (PFA) in PBS, each at room temperature for 20 min. *In situ* hybridization was carried out under the following conditions: probe concentration, 2 μg/ml; hybridization time, 14–18 h; reaction temperature, 42°C.[8, 22]

Immunocytochemistry For comparison with data obtained by *in situ* hybridization at the mRNA level, immunocytochemistry of the cells and tissues was performed. As the first Ab, MAb MRK16 which recognizes the product of MDR1 gene (P-glycoprotein) was utilized.[3, 21] The staining procedure was described elsewhere.[3]

RESULTS

Determination of optimal conditions for dot blot and *in situ* hybridization First, we examined the length of T-T

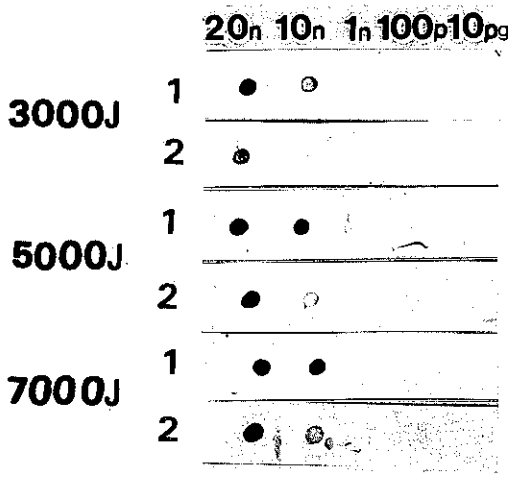

Fig. 1. Immunocytochemical detection of T-T dimerized ss DNA probe on nitrocellulose membrane to assess detection sensitivity by anti-T-T dimer antibody. 1: M13mpM1. 2: M13mp19. 3,000J=3,000 J/m^2. 5,000J=5,000 J/m^2. 7,000J=7,000 J/m^2.

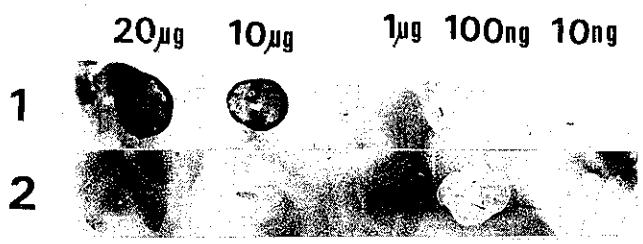

Fig. 2. Dot blot hybridization of total RNAs from K562/ADM using T-T dimerized ss DNA probes. 1: M13mpM1. 2: M13mp19.

dimerized DNA after treatment with S1-nuclease. The average length of the probe DNAs (M13mpM1 and M13mp19) was in the range of 200 to 300 bases (data not shown). It difficult to obtain probe DNAs with less than 300 bases by sonication (data not shown). Next, we carried out experiments for optimizing T-T dimerization of the DNA probes used. Fig. 1 shows that the optimal UV dose for the DNA probes was 5,000 J/m^2 or 7,000 J/m^2 when it was determined by a dot blot hybridization method. In this experiment, disks with 1 ng or more of T-T dimeridized DNA were stained. We also performed dot blot hybridization using T-T dimerized DNA to see whether the T-T dimerized DNAs hybridized with cellular RNA from P-glycoprotein-expressing K-562/ADM or not. As shown in Fig. 2, the spots with more than 10 μg of M13mpM1 were clearly stained, but the spots with M13mp19 DNA were not stained. Lastly, we examined

Table I. Immunocytochemical Staining and *in situ* Hybridization at the mRNA Level

Tissue or cell	Reactivity of MRK 16 MAb[a)]	*In situ* hybridization[b)]
Fetal adrenal	−	−
Fetal kidney	+	+ (proximal renal tubules)
Fetal lung	−	−
Fetal liver	−	−
Fetal spleen	−	−
Fetal stomach	−	−
Fetal intestine	−	−
Sc2 (gastric carcinoma)	+	+
K-562/ADM	+	+
K-562	−	−
CEM	−	−
CEM/VLB	+	+
A2780	−	−
2780^{AD}	+	+
KB	−	−
KB-C4	+	+

a) Intensity of immunostaining was classified as follows: −, negative; +, positive.
b) Intensity of immunohistological staining was classified as follows: −, negative; +, positive.

the efficacy of two fixatives, i.e., ethanol/acetic acid (3:1) and 4% PFA, for hybridization. A better signal was obtained with 4% PFA fixation (data not shown).

***In situ* hybridization** As stated in the previous section, we carried out *in situ* hybridization experiments under the following conditions: fixation, 4% PFA in PBS; UV irradiation, 5,000 J/m^2; size of the probe DNAs, 300–400 bases. Table I summarizes the results of the *in situ* hybridization experiments. The four cell lines, K-562/ADM, CEM/VLB, 2780^{AD}, and KB-C4, all possessing P-glycoprotein, had MDR1 mRNA. As shown in Fig. 3A, MDR1 mRNA was found focally in the cytoplasm of K-562/ADM cells. Elevated levels of MDR1 mRNA were not found in any of the K-562/ADM cells. We performed *in situ* hybridization using available freshly frozen sections. MDR1 mRNA was found in cells of proximal renal tubules and gastric cancer cells (Figs. 4 and 5). In Sc2, MDR1 mRNA was abundantly present in the cytoplasm of the cancer cells, but not in the cytoplasm of the surrounding fibroblasts, and Sc2 cancer cells were also stained with MRK 16 MAb reacting with P-glycoprotein (Table I). We could not detect MDR1 mRNA on the cryostat sections when the same fresh-frozen tissue blocks were sectioned in a cryostat more than three times.

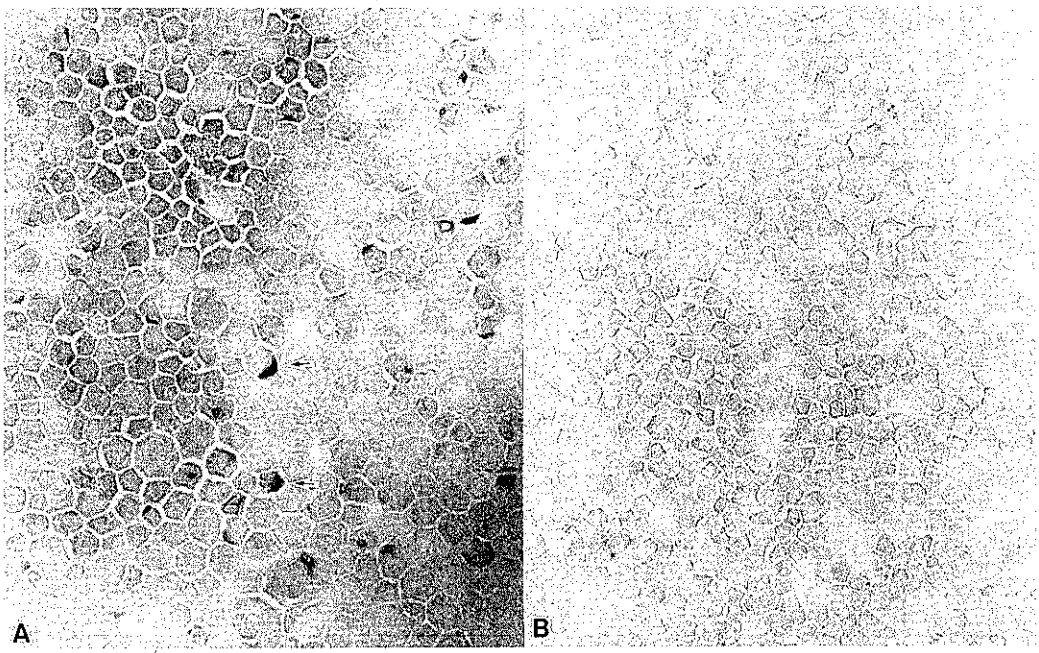

Fig. 3. K-562/ADM cells hybridized *in situ* with T-T dimerized S1 nuclease-treated M13mpM1 (A) or M13mp19 DNA (B). MDR1 mRNA was found focally in the cytoplasm (arrow). Original magnification ×550.

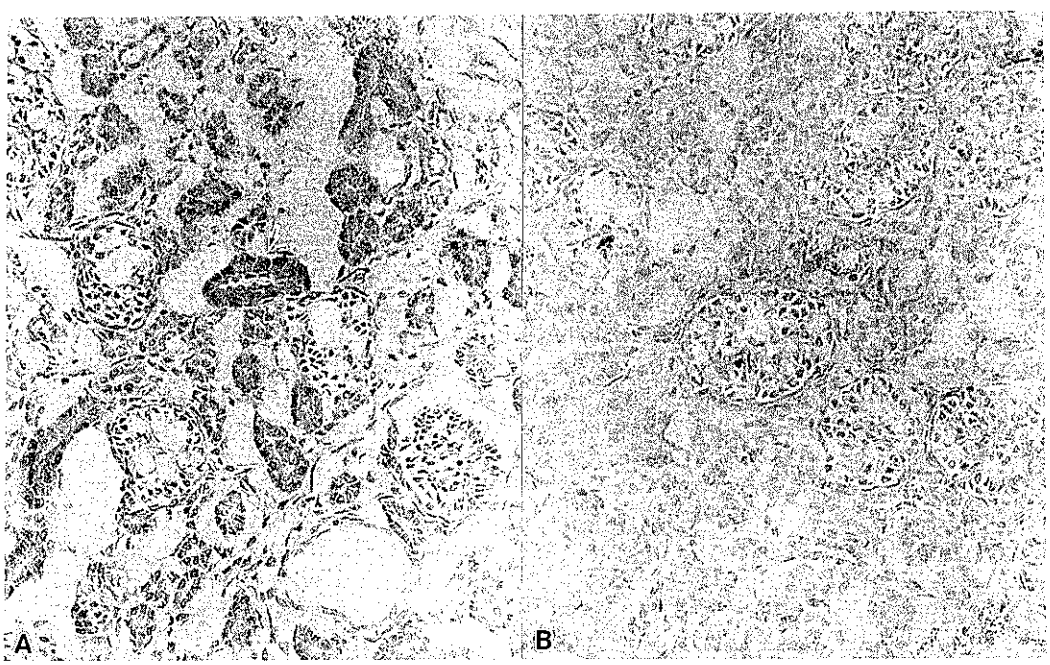

Fig. 4. A freshly frozen section of human fetal kidney hybridized *in situ* with T-T dimerized M13mpM1 (A) or M13mp19 DNA (B). MDR1 mRNA was found in the cytoplasm of cells of proximal renal tubules (arrow). Original magnification ×400.

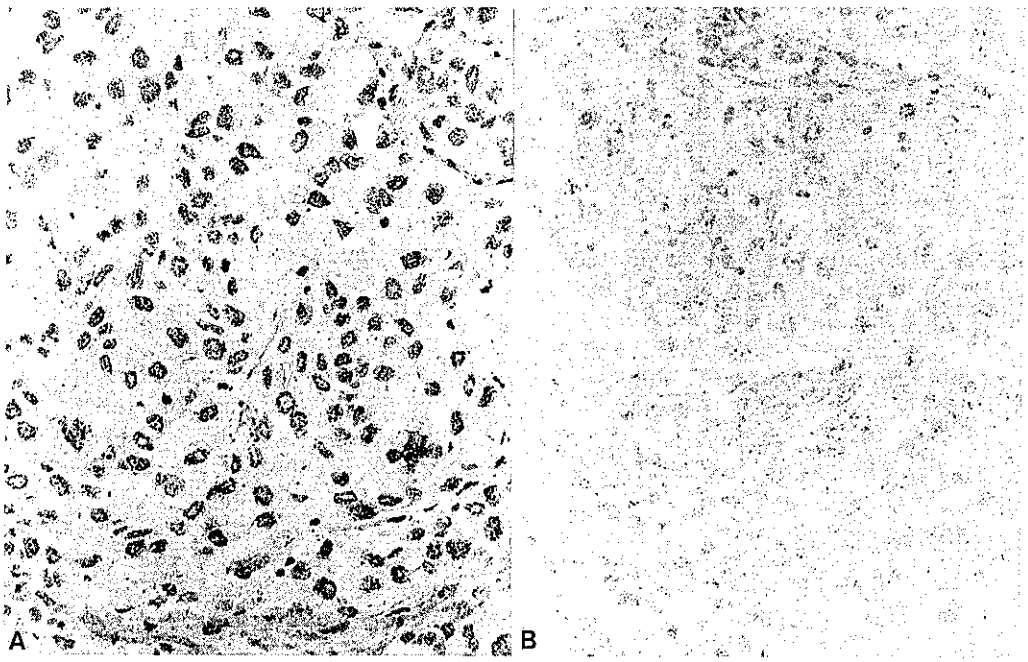

Fig. 5. A freshly frozen section of human gastric carcinoma (Sc2) hybridized *in situ* with T-T dimerized M13mpM1 (A) or M13mp19 DNA (B). MDR1 mRNA was exclusively found in the cytoplasm of the cancer cells (arrow). Original magnification ×400.

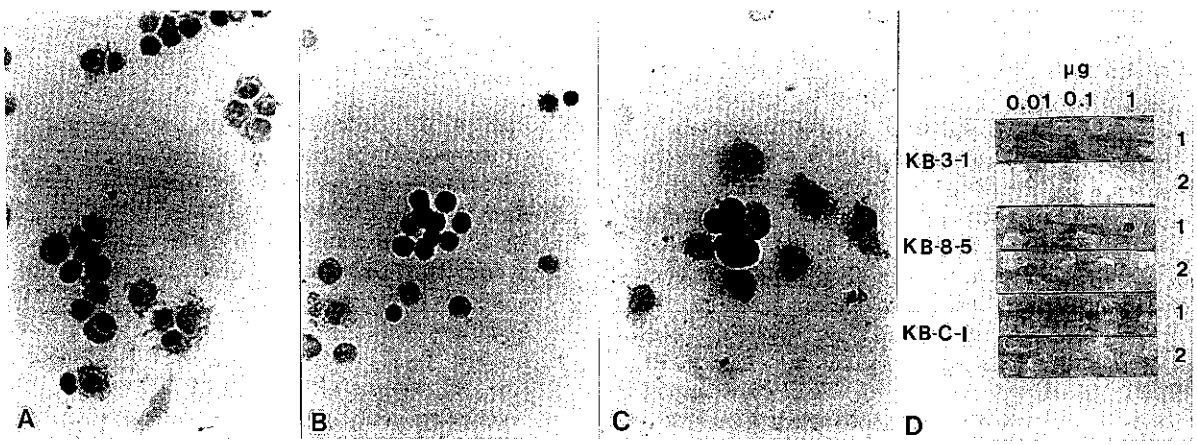

Fig. 6. Immunocytochemistry and dot blot hybridization of KB-3-1, KB-8-5 and KB-C1 immunostained with MRK 16 (10 μg/ml). ABC-PO method. A. KB-3-1 cells. ×400. B. KB-8-5 cells. ×400. C. KB-C1 cells. ×550. D. Dot blot hybridization of the cellular RNA from KB-3-1, KB-8-5 and KB-C1 cells. 1, M13mpM1 as a probe. 2, M13mp19 as a negative control probe.

Dot blot hybridization In order to examine the sensitivity of our methodology, we used three cell lines with different expressions of MDR1 mRNA. As shown in Fig. 6, KB-C1 had the most elevated expression of MDR1 mRNA, and KB-8-5 had an intermediate level of MDR1 mRNA, while the drug-sensitive parental cell line KB-3-1 hardly showed MDR1 mRNA expression. These different expressions of MDR1 mRNA correlated well with expression of P-glycoprotein, the MDR1 gene product (Fig. 6).

DISCUSSION

We could successfully localize MDR1 mRNA in cells as well as tissues by *in situ* hybridization using T-T dimerized ss DNA Probes. Our method has several advantages over those using other ligands as non-radioactive probes,[25-27] as stated by Nakane *et al.*[8] In this study, we improved the previous method by utilizing ss DNA probes to avoid the decrease of the effective concentration of probe DNA during hybridization when double-stranded DNA is used as a probe. We treated T-T dimerized DNA probes with S1 nuclease to obtain short (300–400 bases) T-T dimerized DNA fragments that could easily penetrate into the cytoplasm and hybridize with mRNA. The use of S1 nuclease is especially convenient for short DNAs. We were not able to obtain DNA probes less than 500 bases long by sonication, even though DNA was sonicated for a long period of time (data not shown).

It has recently been reported that there is some heterogeneity in expression of the MDR1 gene in colchicine-resistant KB cells on the basis of an *in situ* hybridization analysis with ^{35}S-labeled RNA probes.[7] However, great care will be needed in interpreting the results because of the background of grains on MDR1 mRNA-negative cells.[7] On the other hand, our method clearly and differentially detects MDR1 mRNA in cells with different expressions of MDR1 mRNA. We also noted heterogeneity in expression of the MDR1 gene in K562/ADM cells. Therefore, using this technique, it may be possible to analyze small samples of tumor cells for amount and heterogeneity of MDR1 gene expression. The possibility that the heterogeneity that we saw may reflect variations in our technique can not be completely ruled out.

Much attention should be paid to preserving tissues. We used malignant tissues possessing P-glycoprotein recognized by MAb MRK 16 which were frozen one hour after extirpation. MDR1 mRNA was found in cytoplasm by this technique (data not shown). The tissues should be frozen in pieces as soon as possible after surgery. We have recently found that P-glycoprotein was highly expressed in untreated lung and in breast cancer, and that P-glycoprotein was expressed in adult adrenal but not in fetal adrenal.[3,4]

It will be interesting to confirm that cell types in these tissues are expressing the MDR1 mRNA. This information will also be helpful in designing more rational chemotherapy protocols.

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