UC 577,1; 61 ISSN 0354-3447

Jugoslov Med Biohem 23: 127–133, 2004 *Originalni naučni rad*
Original paper

GENE EXPRESSION ANALYSIS BY NON-RADIOACTIVE RNA-RNA *IN SITU* HYBRIDIZATION TECHNIQUES

Danijela Drakulić, Milena Stevanović, Gordana Nikčević

Institute of Molecular Genetics and Genetic Engineering,
Vojvode Stepe 444a, P.O. BOX 446, 11001 Belgrade, Serbia and Montenegro

Summary: RNA-RNA *in situ* hybridization is a reliable method for studying tissue and cell specific gene expression, which enables visualization of labeled antisense RNA probe hybridized to specific mRNA. In this study we employed non-radioactive RNA-RNA *in situ* hybridization using biotin- or digoxigenin-labeled RNA probes in order to detect *SOX* gene expression in carcinoma cell lines. By this approach we confirmed results obtained by Northern blot analysis, where the presence of *SOX2* mRNA in NT2/D1 and *SOX14* mRNA in HepG2 cells has been established. Our aim was to set up RNA-RNA *in situ* hybridization method in *in vitro* cultured cells in order to perform further analyses of *SOX* gene expression on various normal and cancer tissues.

Key words: in situ hybridization, non-radioactively labeled RNA probes, human *SOX* genes, carcinoma cell lines

Introduction

The *in situ* hybridization techniques are a powerful tool for studying tissue and cell specific gene expression (1). The use of this method for the detection of messenger RNA molecules is increasing in recent years, and it has a broad application in basic science and diagnostic clinical research (2).

RNA-RNA *in situ* hybridization is based on the ability of RNA molecules to form stable hybrids. The non-radioactively labeled single-stranded probes of antisense RNA are generated by *in vitro* transcription and hybridized to cellular mRNA (1). This type of hybrids is particularly stable and allows stringent washing conditions resulting in highly specific signal with low background (3). Therefore, RNA-RNA *in situ* hybridization is preferable in detection of mRNA in cells compared to DNA-RNA *in situ* hybridization.

Non-radioactively labeled probes are widely used and non-radioactive *in situ* hybridization is a method of preference compared with the radioactive one. It is safe, probes can be stored for long periods without loss of activity, the hybridization sites can be detected quickly and by using different probe labels it is possible to detect several sequences simultaneously (4).

The *SOX* genes comprise a family of transcriptional regulators (5). They function both as classical transcription factors and as modulators of chromatin structure (6). Expression patterns and functional analyses suggest that Sox proteins are involved in control of nervous system development, lens development, chondrogenesis and sex determination (7).

Recently, there is an increasing evidence that *Sox* genes have oncogenic potential. For example, human *SOX7* mRNA was significantly up regulated in pancreatic cancer cell lines, as well as in some cases of primary gastric cancer, and it was significantly down regulated in several cases of primary colorectal, breast, kidney, lung, and prostate cancer (8). Xia et al. (9) reported that overexpression of *cSox3* from retroviral vector transfected in chicken embryo fibroblasts induced oncogenic transformation and aberrant cell growth. Furthermore, loss-of-function mutations of *Xenopus Sox17α*, *Sox17β*, *Sox3*, and mouse *Sox7*, which are all reported to be negative regulators of the WNT-β-catenin-TCF pathway, lead to carcinogenesis (10). Also, it was reported that human *SOX1* and *SOX2* are immunogenic tumour antigens found in small cell lung cancer (11), while human *SOX10* encodes a melanoma/melanocyte differentiation antigen recognized by CTLs (12).

Address for correspondence
Gordana Nikčević
Institute of Molecular Genetics and Genetics Engineering
Tel.: +381 11 3976 212; fax +381 11 3975 808;
e-mail: gordnik@eunet.yu

The aim of this study was to analyze *SOX* gene expression by non-radioactive RNA-RNA *in situ* hybridization in human carcinoma cell lines. Precisely, we examined *SOX2* and *SOX14* mRNA in NT2/D1 human embryonal carcinoma cell line and in hepatocellular carcinoma cell line (HepG2), respectively. Our goal was to set up the method in cell lines in order to test human *SOX* gene expression in tumour and normal tissues, and explore the possibility if this family of genes was involved in tumourogenesis.

Materials and Methods

Plasmid DNA

DNA fragments, which served as templates for *in vitro* transcription in our study, were incorporated into transcription vector pBluescriptT II KS+ phagemid (Stratagene Cloning Systems, San Diego, CA). These inserts are: FbCl2 clone which contains complete ORF of human *SOX2* gene (13) and P3*Pst*I clone which contains 700bp *SOX14* gene fragment, encompassing HMG-box (14).

Both plasmids were purified by Wizard minipreps DNA purification system (PROMEGA).

Cell culture

The NT2/D1 cells (ATCC Number: CRL-1973) and HepG2 cells (ATCC Number: HB 8065) were maintained in DMEM (Gibco BRL), supplemented with 10% foetal bovine serum (Gibco BRL). 1× MEM Non-essential Amino Acid (SIGMA) was added to HepG2 growth medium. Cells were incubated at 37 °C in either a 10% CO_2 atmosphere (NT2/D1 cells) or 5% CO_2 atmosphere (HepG2 cells), allowed to grow near confluency and harvested by trypsin. Cells were then pelleted by centrifugation and resuspended in fresh growth medium. Viable cell counts were determined by trypan blue staining and 1×10^5 cells were seeded per 24×24 mm poly-L-lysine treated coverslips. Coverslips with the cells were then placed in a 60 mm tissue culture dishes, incubated for 1 hour at 37 °C in order to allow cell attachment and finally 3 mL of the growth medium was added to dishes. Cells were incubated overnight at 37 °C in appropriate CO_2 atmosphere.

In vitro transcription

FbCl2 was linearized with *Pst*I restriction enzyme for both sense and antisense synthesis. P3*Pst*I was linearized with *Eco*RI (antisense) and *Bam*HI (sense). Linearized plazmids were then treated with 0.5% SDS and 100 μg/mL proteinase K at 50 °C for 30 minutes and were purified by phenol-chloroform extraction and ethanol precipitation.

The transcription was performed in 20 μL reaction containing 1 μg linearized plasmid (sense or antisense), 2.5 mmol/L each rATP, rCTP, rGTP, 1.5 mmol/L rUTP, 0.5 mmol/L biotin-16-uridin-5'-triphosphate or 0.5 mmol/L digoxigenin-11-uridine-5'-triphosphate, 1×Transcription Buffer, 10 mmol/L DTT, 40 U Recombinant RNasin Ribonuclease Inhibitor and 20 U of either T7 or T3 RNA polymerase. Control *in vitro* transcription reaction was performed with rNTPs, 2.5 mmol/L each. The reaction was incubated 2 hours at 37 °C and after that template DNA was removed by adding 2U RNase-free DNaseI.

The reaction was stopped by 1 μL 0.5 mol/L EDTA. G-50 spin columns and precipitation were used in order to remove free nucleotides from the reaction.

The quality of biotin- and digoxigenin (DIG)-labeled RNA probes was checked on 1% agarose gel.

In situ hybridization

The probe mixture contained 50 ng probe, 6 μg of salmon sperm DNA, and 3 μg tRNA was dried down in a vacuum centrifuge, resuspended in 10 μL deionized formamide and then denatured by boiling for 10 min. Then, equal volume of hybridization buffer (4×SSC, 0.02 mol/L DTT and 20% dextran sulfate) was added to probe mixture.

The cells on coverslips were washed in phosphate buffered saline (PBS; pH 7.4), fixed in 4% paraphormaldehyde/PBS-$MgCl_2$ for 10 min at room temperature, followed by addition of cold 70% ethanol and placed at –20 °C until use. The cells were rehydrated in Tris-HCl/glycine and prehybridized in 50% deionized formamide/4×SSC at 65 °C for 10 min. After the prehybridization coverslips were inverted onto 20 μL droplets of probe on parafilm, covered with second sheet of parafilm and incubated over night in hybridization buffer at 42 °C.

Following hybridization, the coverslips were incubated at 37 °C on droplets of prewarmed RNase solution (40 μg/mL RNase A, 2 μg/mL RNase T1, 10 mmol/L Tris-HCl pH 7.5, 5 mmol/L EDTA, 0.3 mol/L NaCl) to remove unhybridized probe. Then, coverslips were washed with agitation at 37 °C in 2×SSC for 30 minutes, in 1×SSC for 15 minutes and in 0.5×SSC for 15 minutes.

Detection of biotin-labeled RNA probes. Detection was performed as described (15). The biotinylated probes were detected with avidin-DCS conjugated to FITC and biotinylated anti-avidin DCS (Vector Laboratories). For amplification of signals four layers of avidin-FITC were used.

The slides were mounted on 0.4 mg/mL DAPI (Diamidino phenylindole) and Propidium Iodide, counterstain in Vectashield Antifade buffer, and then viewed under Olympus RFL BH2 epifluorescent microscope equipped with barrier filter for FITC (O 515). The slides were photographed using Fuji 100 color film.

Detection of digoxigenin-labeled RNA probes. The coverslips were washed for 5 minutes at room

temperature with 100 mmol/L Tris-HCl pH 7.5/150 mmol/L NaCl and then for 30 minutes at room temperature with blocking buffer (100 mmol/L Tris-HCl pH7.5/150 mmol/L NaCl/saturated with blocking reagent). Then, coverslips were incubated for 30 minutes at 37 °C with anti-DIG-alkaline phosphatase (Fab fragments), and then coverslips were washed at room temperature for 10 minutes with 100 mmol/L Tris-HCl pH7.5/150 mmol/L NaCl and for 10 minutes with detection buffer (Tris-HCl pH 9.5/100 mmol/L NaCl/50 mmol/L $MgCl_2$). The coverslips were covered with detection buffer containing 0.18 mg/mL BCIP (5-bromo-4-chloro-3-indolyl phosphate), 0.34 mg/mL NBT (nitroblue tetrazolium) and 240 μg/mL levamisole and after 1 hour the color was developed. Levamisole is included in the substrate solution in order to inactivate the endogenous activity of alkaline phosphatase, since this enzyme is part of the DIG-detection system (2). The color reaction was stopped with 10 mmol/L Tris pH 8.0/1 mmol/L EDTA. The coverslips were mounted with Kaiser's solution (Merck) and were viewed under Olympus B×51 microscope.

Results and Discussion

In order to analyze *SOX2* and *SOX14* gene expression in carcinoma cell lines we performed RNA-RNA *in situ* hybridization experiments using non-radioactively labeled RNA probes. To check accurate length and integrity of RNA probes after in vitro transcription we run probes on agarose gels. One example is illustrated in *Figure 1*. This experiment confirmed that newly synthesized RNA probes were intact and of correct length (*Figure 1 lane 3* and *Figure 1 lane 5*), therefore, could be used for *in situ* hybridization.

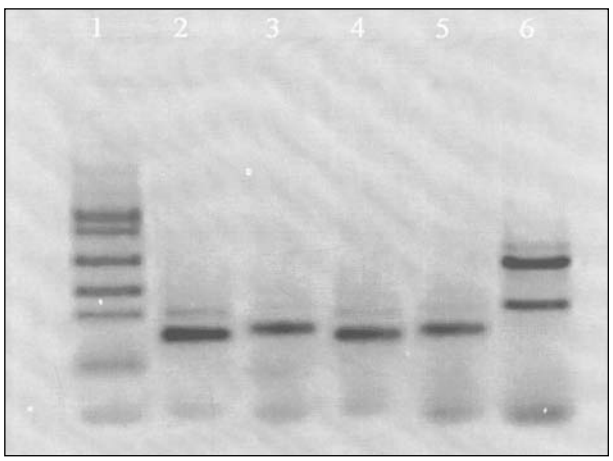

Figure 1. *SOX14* RNA probes synthesized by *in vitro* transcription.
Lane 1: RNA ladder. Starting at the bottom of the gel the size markers are 0.24, 1.35, 2.37, 4.40, 7.46, 9.49 Kb
Lane 2: unlabeled antisense probe
Lane 3: digoxigenin-labeled antisense probe
Lane 4: unlabeled sense probe
Lane 5: digoxigenin-labeled sense probe
Lane 6: total human RNA
As expected, digoxigenin-labeled RNA probes migrate through gel more slowly than unlabeled RNA probes.

Analysis of SOX2 gene expression using biotin- and DIG-labeled probes

Sox2 are predominantly expressed in the immature, undifferentiated cells of the neural epithelium of the entire CNS (16, 17) and the switch from proliferating to differentiating cells correlates with the decrease in *Sox2* expression (17). Also, it has been shown that constitutive expression of *SOX2* inhibits neuronal differentiation and results in the maintenance of progenitor characteristics (18).

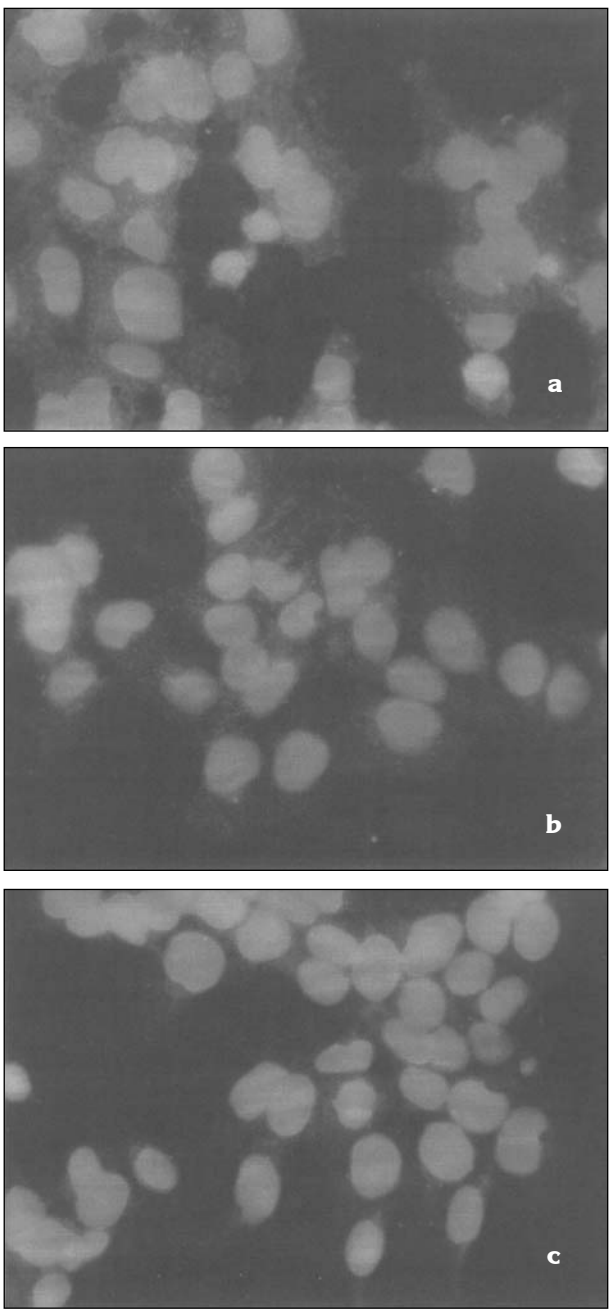

Figure 2. *In situ* hybridization to SOX2 mRNA in *in vitro* cultured NT2/D1 cells with biotin-labeled RNA probes:
a) Antisense probe; b) Sense probe; c) No probe

It is well known that cancer cells are usually closer in their properties to immature normal cells than to more mature cell types (19). Therefore, for *SOX2* gene expression analysis we decided to use human embryonal carcinoma cell line, NT2/D1. This cell line phenotypically is an undifferentiated, pluripotent embryonic stem cells (20), and thus is an excellent *in vitro* model for studying *SOX2* gene expression.

We started our analysis of *SOX2* mRNA in NT2/D1 cells by using biotin-labeled probes. The length and quality of *in vitro* transcribed *SOX2* antisense and sense RNA probes were examined on agarose gel (data not shown). We continued our study by performing *in situ* hybridization using antisense *SOX2* RNA probe, as well as appropriate controls (*Figure 2*). Strong hybridization signal (bright green) is detected in cytoplasm when an antisense probe was used (*Figure 2a*). However, although much weaker, signals are visible in negative controls. Precisely, in cells when the sense probe was used as a control (to rule out nonspecific binding, *Figure 2b*), as well as in cells without probe (to rule out endogenous biotin, *Figure 2c*); background signals are also less abundant, but still present. This approach revealed the presence of *SOX2* mRNA in NT2/D1 cells which correlates with results obtained by Northern blot analysis (21).

The background signal seen in negative controls (*Figure 2b* and *2c*) could be explained by the endogenous biotin naturally present in almost every tissue and cell (22). It is well known for example, that liver is very rich in endogenous biotin (22) and since we wanted to include hepatocellular carcinoma cell line (HepG2) in our study we decided to continue our *in situ* hybridization analysis by using DIG-labeled RNA probes.

The DIG-labeling method is based on a steroid isolated from digitalis plants. In contrast to biotin which is present in almost every tissue and cell, the blossoms and the leaves of digitalis plants are the only natural source of digoxigenin, indicating that anti-DIG antibody will not bind to other biological material (23).

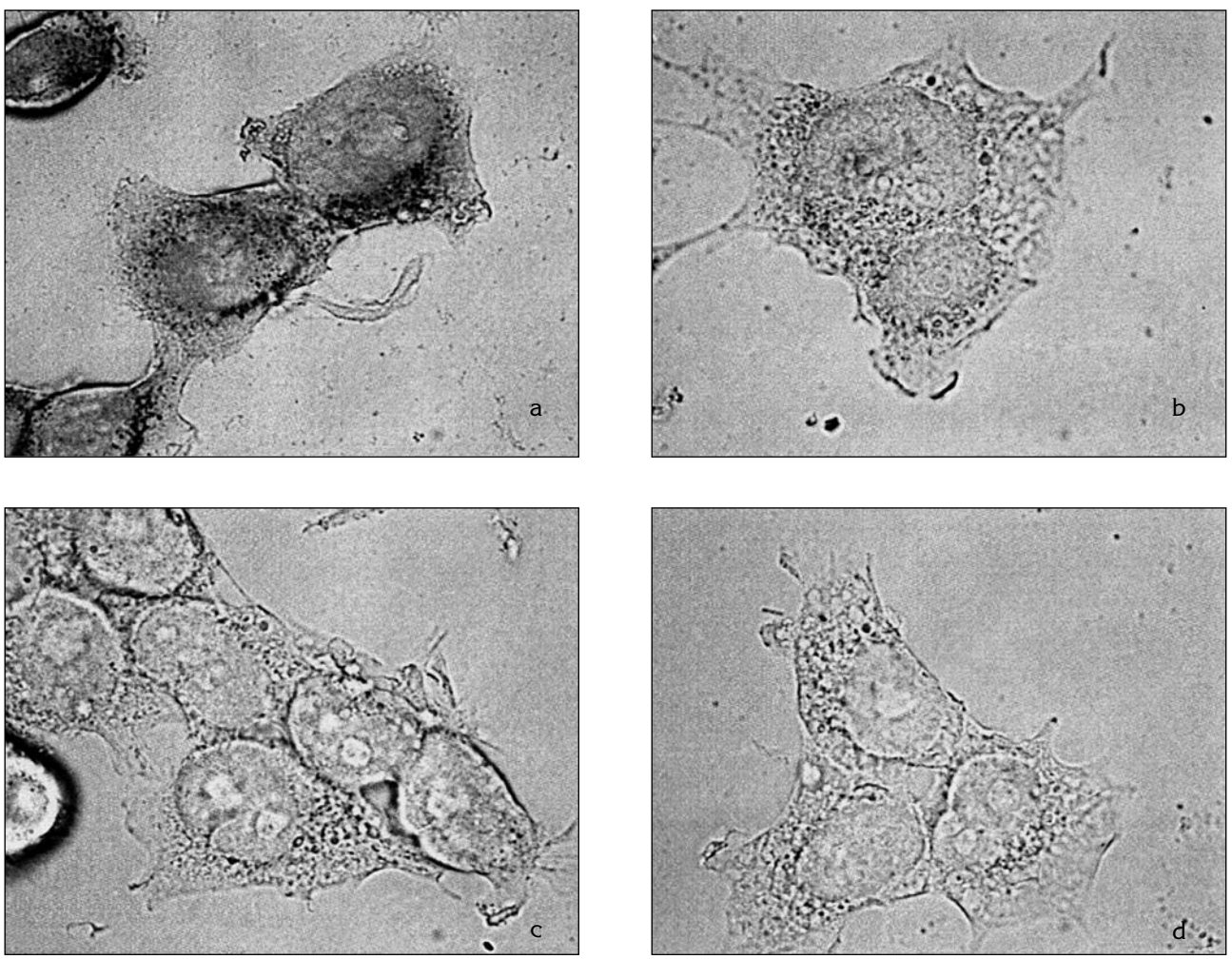

Figure 3. *In situ* hybridization to SOX2 mRNA in *in vitro* cultured NT2/D1 cells with digoxigenin-labeled RNA probes: a) antisense probe; b) sense probe; c) no probe; d) antisense probe with no antibody

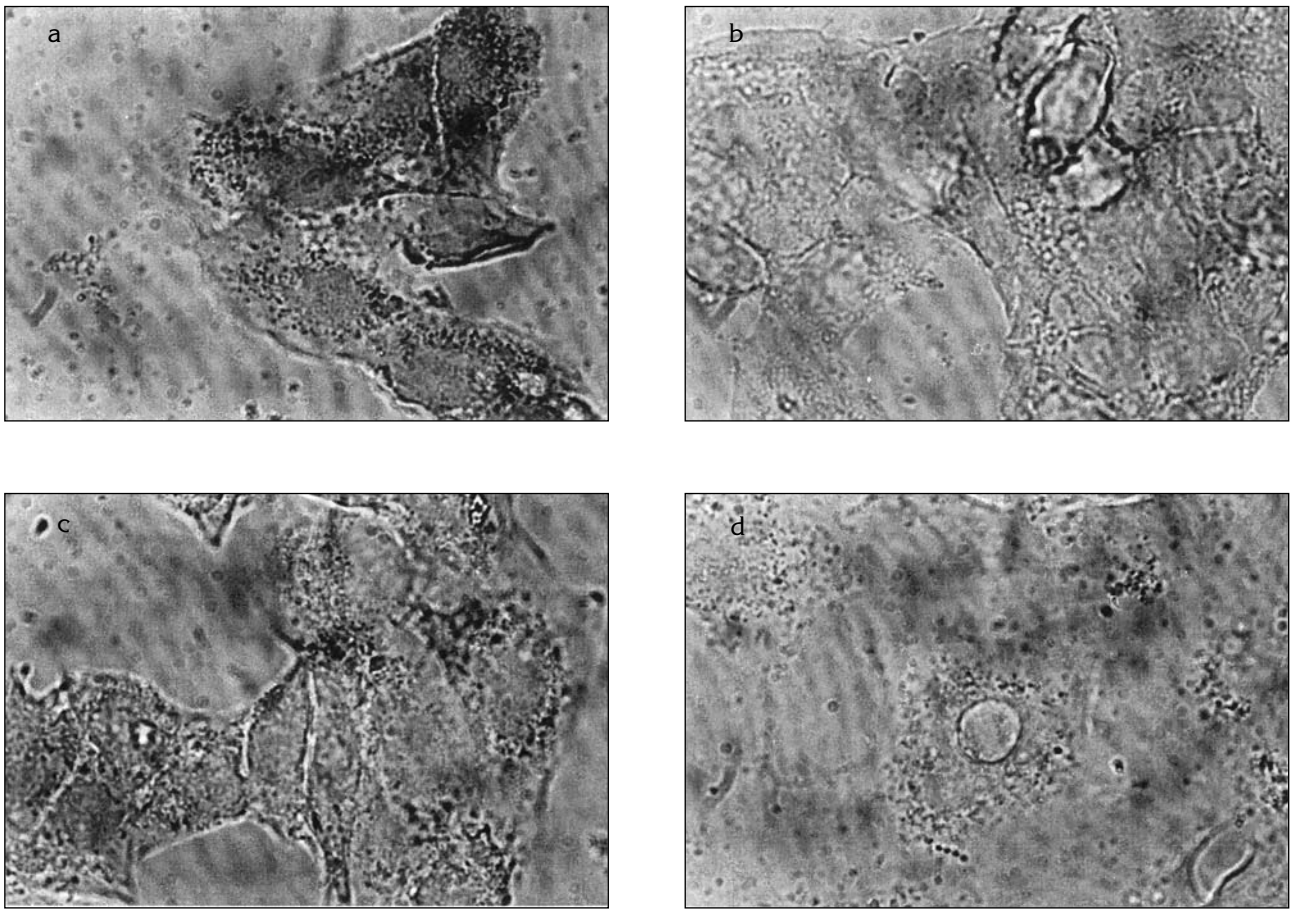

Figure 4. *In situ* hybridization to SOX14 mRNA in *in vitro* cultured HepG2 cells with digoxigenin-labeled RNA probes: a) antisense probe; b) sense probe; c) no probe; d) antisense probe with no antibody

Firstly, we have checked newly synthesized DIG-labeled *SOX2* probe for its integrity and length uniformity (data not shown) and then proceeded with *in situ* hybridization. The presence of the *SOX2* mRNA was detected as a dark stain in cytoplasm after the hybridization to antisense RNA probe (*Figure 3a*). All negative controls were processed alongside with the samples: sense probe (*Figure 3b*) showed minimal background; no probe (*Figure 3c*) and control without antibody (*Figure 3d*) showed no detectable signal within the cell. Results presented in *Figure 3* also clearly show expression of *SOX2* gene in NT2/D1 cells.

Analysis of SOX14 gene expression using DIG-labeled probes

It has been reported that human *SOX14* gene is widely expressed and its transcripts were detected by RT-PCR in foetal brain, spinal cord and thymus (14). No visible RT-PCR product was seen in adult liver RNA. Southern blot analysis of the same gel gave very faint signal on liver RNA indicating very low level of *SOX14* gene expression in normal adult liver (14). However, by Northern blot analysis *SOX14* transcript was detected in hepatocellular carcinoma cell line, HepG2 (14) indicating that *SOX14* expression might be up regulated in this carcinoma cell line, which we used as a model in our study.

The probes used in this set of experiments were shown in *Figure 1.* After the hybridization to antisense RNA probe (*Figure 4a*) we detected the *SOX14* mRNA as a dark stain in cytoplasm. As in previous experiment, sense probe (*Figure 4b*) showed minimal background; no probe (*Figure 4c*) and control without antibody (*Figure 4d*) demonstrated no visible signal within the cell. This method revealed the presence of *SOX14* mRNA in HepG2 cells.

Using this approach we confirmed the results obtained by Northern blot analysis, where the presence of *SOX2* mRNA in NT2/D1 (21) and *SOX14* mRNA in HepG2 cells (14) has been established. Our goal was to set up RNA-RNA *in situ* hybridization method in *in vitro* cultured cells in order to further analyze *SOX* gene expression on numerous normal and malignant tissues.

RNA-RNA *in situ* hybridization is a very reliable method, which enables visualization of hybridization to specific mRNA while areas without hybridization appear blank (or unstained). This characteristic is particularly advantageous for tissue gene expression analysis. Precisely, tissues are a heterogeneous population of cells, and gene expression results obtained by Northern or RT-PCR methods reflect the whole tissue rather than a precise cell specific gene expression. Furthermore, more tissue is necessary for Northern analysis (in order to isolate RNA) than for *in situ* hybridization analysis. On the other hand, the smallest amount of tissue is sufficient for a successful RT-PCR (theoretically it is possible to detect only one mRNA molecule). Nevertheless, the gene expression level could not be deduced by using this method since it involves many DNA multiplications.

Therefore, RNA-RNA *in situ* hybridization is a very suitable approach that would allow us to perform analysis of *SOX* gene expression in various normal and cancer tissues.

In conclusion, we have employed non-radioactive RNA-RNA *in situ* hybridization where biotin- or digoxigenin-labeled RNA probes were used to detect *SOX* gene expression in carcinoma cell lines. If our research confirms that *SOX* gene expression is modified in neoplastic transformation, based on that finding it would be possible to develop diagnostic tests that could be carried out in oncology.

Acknowledgements. This work was supported by the Ministry of Science, Technology and Development, Republic of Serbia (Grant 1438-Human *SOX* genes: studying the structure and regulation of expression).

ANALIZA EKSPRESIJE GENA PRIMENOM NERADIOAKTIVNE RNK-RNK *IN SITU* HIBRIDIZACIJE

Danijela Drakulić, Milena Stevanović, Gordana Nikčević

Institut za molekularnu genetiku i genetičko inženjerstvo, Beograd

Kratak sadržaj: RNK-RNK *in situ* hibridizacija je pogodna metoda za izučavanje specifične ekspresije gena u tkivima i ćelijama, kojom je omogućena vizualizacija obeležene antisens RNK probe hibridizovane za specifičnu iRNK. U ovom radu prikazano je detektovanje ekspresije *SOX* gena u tumorskim ćelijskim linijama primenom neradioaktivne RNK-RNK *in situ* hibridizacije koristeći RNK probe obeležene biotinom ili digoksigeninom. Koristeći ovaj pristup potvrđeni su rezultati dobijeni Northern blot analizom, tj. pokazano je prisustvo *SOX2* iRNK u NT2/D1 i *SOX14* iRNK u HepG2 ćelijama. Cilj ovog rada bio je uspostavljanje RNK-RNK *in situ* hibridizacije na *in vitro* kultivisanim ćelijama da bi metodu primenili pri proučavanju ekspresije *SOX* gena u različitim normalnim i tumorskim tkivima.

Ključne reči: in situ hibridizacija, neradioaktivno obeležene RNK probe, *SOX* geni, karcinoma ćelijske linije

References

1. Höfler H. Principles of in situ hybridization. In: Polak JM, McGee JO'D. *In situ* hybridization-principles and practice. New York: Oxford University Press, 1990: 15–29.
2. Dijkman HBPM, Mentzel S, de Jong AS, Assmann KJM. RNA *in situ* hybridization using DIG-labeled cRNA probes. Biochemica (U.S. version) 1995; 2: 23–7.
3. Komminoth P. Detection of mRNA in tissue sections using DIG-labeled RNA and oligonucleotide probes. In: Roche Applied Science. Nonradioactive *In Situ* Hybridization Application Manual. http://www.roche-applied-science.com/, 2003: 149–63.
4. Leitch AR, Schwarzacher T, Jackson D, Leitch IJ. RMS Microscopy Handbook vol. 27: *In situ* Hybridization: a practical guide. 1st ed. BIOS Scientific Publishers, 1994: 36–8.
5. Pevny LH, Lovell-Badge R. *Sox* genes find their feet. Curr Opin Genet Dev 1997; 7: 338–44.
6. Wilson M, Koopman P. Matching *SOX*: partner proteins and co-factors of the *SOX* family of transcriptional regulators. Curr Opin Genet Dev 2002; 12: 441–6.
7. Wegner M. From head to toes: the multiple facets of Sox proteins. Nucleic Acid Res 1999; 27: 1409–20.
8. Katoh M. Expression of human *SOX7* in normal tissue and tumors. Int J Mol Med 2002; 9: 363–8.
9. Xia Y, Papalopulu N, Vogt PK, Li J. The Oncogenic potential of the High Mobility Group Box Protein Sox3. Cancer Res 2000; 60: 6303–6.
10. Saitoh T, Katoh M. Expression of human *SOX18* in normal tissues and tumors. Int J Mol Med 2002; 10: 339–44.
11. Güre AO, Stockert E, Scanlan MJ, Keresztes RS, Jäger D, Altorki NK, et al. Serological identification of embryonic neural proteins as highly immunogenic tumor antigens in small cell lung cancer. Proc Natl Acad Sci USA 2000; 97: 4198–203.
12. Khong HT, Rosenberg SA. The Waardenburg syndrome type 4 gene, *SOX10*, is a novel tumor-associated antigen identified in a patient with a dramatic response to immunotherapy. Cancer Res 2002; 62: 3020–3.
13. Stevanović M, Zuffardi O, Collignon J, Lovell-Badge R, Goodfellow P. The cDNA sequence and chromosomal location of the human *SOX2* gene. Mamm Genome 1994; 5: 640–2.
14. Arsić N, Rajić T, Stanojčić S, Goodfellow PN, Stevanović M. Characterization and mapping of the human *SOX14* gene. Cytogenet Cell Genet 1998; 83:139–46.
15. Wilkinson DG. *In situ* hybridization to chromosomes with biotinylated probes. In: Rickwood D, Hames DB. eds. *In Situ* Hybridization-A Practical Approach. IRL Press: Oxford University Press, 1995: 137–56.
16. Collignon J, Sockanathan S, Hacker A, Cohen-Tannoudji M, Norris D, Rastan S, et al. A comparison of the properties of *Sox3* with *Sry* and two related genes, *Sox1* and *Sox2*. Development 1996; 122: 509–20.
17. Uwanogho D, Rex M, Cartwright EJ, Pearl G, Healy C, Scotting PJ, et al. Embryonic expression of the chicken *Sox2, Sox3* and *Sox11* genes suggests an interactive role in neuronal development. Mech Dev 1995; 49: 23–36.
18. Graham V, Khudyakov J, Ellis P, Pevny L. *Sox2* function to maintain neural progenitor identity. Neuron 2003; 39: 749–65.
19. Cancer: Characteristics of tumor cells. In: Lodish H, Baltimore D, Berk A, Zipursky SL, Matsudaira P, Darnell J Molecular Cell Biology. 3rd ed. Freeman HW and Company, New York: Scientific American Books, 1995: 1248–51.
20. Andrews PW, Damjanov I, Simon D, Banting G, Carlin C, Dracopoli NC, et al. Pluripotent embryonal carcinoma clones derived from the human teratocarcinoma cell line TERA-2: differentiation *in vivo* and *in vitro*. Lab Invest 1984; 50: 147–62.
21. Stevanović M. Modulation of *SOX2* and *SOX3* gene expression during differentiation of human neuronal precursor cell line NTERA2. Mol Biol Rep 2003; 30: 127–32.
22. Dix DJ, Eisenberg BR. *In situ* hybridization and immunocytochemistry in serial sections of skeletal muscle to detect myosin expression. J Histochem Cytochem 1988; 36: 1519–26.
23. Introduction to Hapten Labeling and Detection of Nucleic Acids. In: Roche Applied Science. Nonradioactive *In Situ* hybridization Application Manual. http://www.roche-applied-science.com/, 2003; 1: 11.

Received: December 29, 2003

Accepted: February 16, 2004