Kvantitativna *in situ* hibridizacija povećane osjetljivosti u mekom, koštanom i zubnom tkivu pomoću RNA probi označenih digoksigeninom

Quantitative *in situ* hybridization with enhanced sensitivity in soft, bone and tooth tissue using digoxigenin tagged RNA probes

Jelica Gluhak-Heinrich[1], Wuchen Yang[2], Marie A. Harris[2], Stephen E. Harris[2]

[1]Klinika za ortodonciju, Sveučilište u Texasu, Zdravstveni znanstveni centar u San Antoniju, San Antonio, Teksas, SAD
[1]Department of Orthodontics, The University of Texas Health Science Center at San Antonio, San Antonio, Texas, USA
[2]Klinika za periortodonciju, Sveučilište u Texasu, Zdravstveni znanstveni centar u San Antoniju, San Antonio, Teksas, SAD
[2]Department of Periodontics, The University of Texas Health Science Center at San Antonio, San Antonio, Texas, USA

Sažetak

Uvod: Kvantitativna neradioaktivna *in situ* hibridizacija je moćna tehnika za lokalizaciju ekspresije transkripata mRNA. Ove metode omogućavaju otkrivanje mRNA uz visoku rezoluciju na razini jedne jedine stanice. Dosad najšire opisani neradioaktivni protokoli rabili su deblje kriostatske rezove mekog tkiva i lokalizirali visoko zastupljene gene bez kvantificiranja.

Materijali i metode: Mi smo razvili metodu neradioaktivne *in situ* hibridizacije pomoću tankih rezova demineraliziranog koštanog tkiva uklopljenog u parafin, uz otkrivanje nisko zastupljenih gena i kvantifikaciju *in situ* signala. Naš protokol zasniva se na optimalnoj sintezi digoksigeninom obilježenih DNA probi za vizualiziranje vrlo nisko zastupljenih gena u mekom, koštanom i zubnom tkivu uklopljenom u parafin. Naša nova tehnika prikazuje *in situ* signal uz umnožavanje i pojačanje boje kroz reakciju alkalne fosfataze.

Rezultati: Osjetljivost je na razini radioaktivnih protokola, a rezolucija je na razini pojedine stanice, što je bolje nego kod radioaktivnih protokola. Kvantificiranje *in situ* hibridizacijskog signala, što se ranije radilo pomoću radioaktivnog ili fluorescentnog obilježavanja, sad je moguće s alkalnom fosfatazom pomoću naše tehnike i programa ImageJ.

Zaključak: Prikazani primjeri pokazuju kako slijedeća metoda ima bolju dokazanu rezoluciju i jednaku osjetljivost kao radioaktivno obilježene metode u različitim tkivima s mogućom kvantifikacijom; to pak pokazuje da se ova metoda općenito može rabiti u istraživačkim kao i u kliničkim laboratorijima.

Ključne riječi: kvantitativna *in situ* hibridizacija, digoksigeninske probe, koštano tkivo uklopljeno u parafin

Abstract

Introduction: Quantitative non-radioactive *in situ* hybridization is a powerful technique for localizing the expression of mRNA transcripts. These methods enable mRNAs to be detected with great resolution on a single cell level. Up to date the most published non-radioactive protocols used thick cryostat sections in soft tissue localizing high abundant genes without quantification.

Material and methods: We developed a non-radioactive *in situ* hybridization method using thin sections of demineralized bone paraffin embedded tissue with low abundant gene detection and *in situ* signal quantification. Our protocol is based on the optimal synthesis of digoxigenin labeled RNA probes to visualize very low abundant genes in soft, bone and tooth paraffin embedded tissues. Our new technique visualizes an *in situ* signal with amplification and enhanced color by developing alkaline phosphatase reaction.

Results: The sensitivity is at the level of radioactivity and the resolution is at the level of a single cell, which is better than with radioactivity. Quantification of *in situ* hybridization signal, previously used with radioactive or fluorescent labeling, is now possible with alkaline phosphatase using our technique and ImageJ program.

Conclusion: The presented examples show that the following method has better proven resolution and equivalent sensitivity to radioactive labeled methods in different tissues with quantification ability, thus indicating that this method can generally be used in research and clinical laboratories.

Key words: quantitative *in situ* hybridization, digoxigenin probes, paraffin embedded bone tissue

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Uvod

Pokusi u kliničkim i istraživačkim laboratorijima često zahtijevaju *in situ* lokalizaciju mRNA tkivno specifičnih bioloških biljega i njihovu kvantifikaciju unutar specifičnih stanica tkiva (1,2). Prošlo je 37 godina otkako je objavljen prvi protokol za *in situ* hibridizaciju (3). Od toga vremena objavljeni su brojni radovi o ovoj tehnici, koja se danas uvelike rabi u bazičnim i kliničkim istraživanjima (4-7). Objavljeni su radovi koji izvještavaju kako neradioaktivna *in situ* hibridizacija može pokazivati jednaku osjetljivost kao radioaktivna hibridizacija (8). Idealno bi takve tehnike trebale omogućiti otkrivanje mRNA koja je prisutna u vrlo maloj količini na staničnoj razini, ali s osjetljivošću i kvantifikacijskim mogućnostima radioaktivno obilježenih probi pomoću ^{33}P ili ^{35}S. Neki laboratoriji prednost daju otkrivanju kolorimetrijskim pred fluorescentnim metodama; razlog za to je to što rezultat nije osjetljiv na svjetlo, neće s vremenom izblijediti i nema žurbe sa slikama, koje se isto tako mogu ponovno snimati bez fluorescentnog mikroskopa. Prednost fluorescentnih metoda je sulokalizacija mRNA *in situ*, jer ove metode omogućavaju vrlo dobro otkrivanje višestrukih probi i lako kvantificiranje hibridizacijskog signala. S druge strane, fluorescentna *in situ* hibridizacija se tradicionalno provodila s teškoćama, uključujući nizak signal, visok pozadinski signal i autofluorescenciju, naročito u koštanom tkivu (9).

Većina objavljenih protokola za neradioaktivnu *in situ* hibridizaciju razvijena je uz primjenu debljih kriorezova (10-12) te nekolicina uz uporabu tkiva uklopljenog u parafin (13,14). Taj se pristup primjenjivao za vremenske i prostorne uzorke u mekom tkivu s visoko zastupljenim genima. Osjetljiviji hibridizacijski protokoli i otopine bili su potrebni za signal visoke rezolucije u tankim rezovima koštanog i zubnog tkiva, uz primjenu nisko zastupljenih gena na razini pojedinačne stanice.

Neka od ovih pitanja je, na sreću, riješila pojava sustava pojačavanja signala tiramidom (TSA, PerkinElmer), koji smo mi rabili za povećanje osjetljivosti (15).

U ovom izvješću podrobno opisujemo postupnik za otkrivanje mRNA pomoću pojačavanja signala i tehnika za povećan razvoj boje u usporedbi s dvjema drugim različitim neradioaktivnim tehnikama i jednom radioaktivnom tehnikom *in situ* hibridizacije, koje smo također razvili i primijenili u našem laboratoriju. Ove su tehnike vrlo osjetljive i mogu se primjenjivati na tankim parafinskim rezovima mekog tkiva, kao i demineraliziranog zubnog i koštanog tkiva. Ove tehnike mogu otkriti signale nisko zastupljenih gena s jednakom osjetljivošću kao i radioaktivne metode, ali na razini jedne stanice, što se može kvantificirati.

Introduction

Experiments in clinical and research laboratories often require mRNA *in situ* localization of tissue specific biomarkers and their quantification within specific cells in the tissue (1,2). Thirty-seven years have elapsed since the first protocol for *in situ* was published (3). Since then, numerous papers have been published on this technique, which is now widely applied in basic and clinical research (4-7). There are publications describing that non-radioactive *in situ* hybridization can exhibit equivalent sensitivity as radioactive hybridization (8). Ideally, such techniques would allow for detection of very low abundant mRNAs at the cellular level but with sensitivity and quantification capabilities of radioactive labeled probes ^{33}P or ^{35}S. Several laboratories prefer detection with colorimetric methods rather than fluorescent; the reason is that the result is not sensitive to light, it will not fade away with time, and there is no rush with the pictures which can also be retaken without a fluorescent microscope. The advantage of fluorescent methods is co-localization of mRNA *in situ* since these methods permit very good detection of multiple probes and easy quantification of hybridization signal. On the other hand, fluorescence *in situ* hybridization has traditionally been conducted with difficulties involving low signal, high background, and auto-fluorescence, especially in bone tissue (9).

Most of the published protocols for non-radioactive *in situ* hybridization were developed using thick cryosections (10-12), and few protocols with paraffin embedded tissue (13,14). This approach was applied for temporal and spatial patterns in soft tissue with high abundant genes. For high resolution signal in thin sections of bone tissue and teeth using low abundant genes at a single cell level, more sensitive hybridization protocols and solutions were needed.

Fortunately, the advent of the tyramide signal amplification (TSA, PerkinElmer), a system which we applied to increase sensitivity, has resolved some of these issues (15).

In this report, we provide a detailed protocol for detection of mRNA using signal amplification and enhanced color developing techniques compared to two other different non-radioactive techniques and one radioactive *in situ* hybridization technique also developed and applied in our laboratory. These techniques are very sensitive and can be used on thin paraffin sections of soft as well as demineralized tooth and bone tissues. These techniques can detect signals of low abundant genes with the same sensitivity of radioactive methods but at the level of single cell, which can be quantified.

Materijali i metode

Priprava DNA kalupa bez RNaza

RNA probe za *in situ* hibridizaciju načinjene su transkripcijskom reakcijom *in vitro*. Svaki sklop s različitim cDNA probama uveden u plazmidni vektor za transkripciju *in vitro* sadržavao je promotor T_3, T_7 ili SP_6. Kako bismo svaku probu prepisali samo iz željene cDNA (a ne iz čitavog vektora), naše smo vektore digestirali i napravili linearizirane DNA kalupe.

Za pripravu DNA kalupa bez RNaza željenom je restrikcijskom endonukleazom digestirano otprilike 20 μg plazmidnog sklopa koji je sadržavao cDNA za probu (Slika 1.) uz primjenu 100 μL reakcijskog volumena. Ova je količina bila dostatna da omogući obilježavanje najmanje 10 puta.

Linearizirani kalupi su ekstrahirani pomoću TRIS-HCl zasićenog fenola, pH = 8 (bez RNaza) i kloroform-izoamil alkoholom (49:1) u omjeru 1:1, dva puta, te jedanput samo kloroformom. Vodena faza je precipitirana pomoću 3 M natrij acetata, pH = 5,5 (konačna molarnost 0,3 M natrij acetata) i 3 volumena 100%-tnog etanola preko noći na –20 °C. Kuglica DNA dobivena centrifugiranjem isprana je ledenim 70% etanolom (razrijeđenim vodom bez RNaza iz 100%-tnog etanola). Na zraku osušena kuglica je rastopljena u 20 μL 10 mM TRIS (pH = 7,5) (bez RNaza) i pohranjena na –20 °C do kasnije uporabe. Izmjerene koncentracije kalupa za probe bile su između 0,5 i 1,5 μg/μL.

Priprava *antisense* i *sense* mRNA probi za *in situ* hibridizaciju

Za svaku probu rabili smo standardnu *in vitro* transkripcijsku reakciju s digoksigeninom UTP (16). Transkripcijska smjesa koju smo pripravili sadržavala je slijedeće komponente: 1 μL inhibitora RNaza (40 U/μL), 3 μL H_2O bez RNaza, obrađene pomoću DEPC (dietil pirokarbonat, Sigma) (17), 4 μL 5X SP_6 ili T_3, T_7 polimerazni pufer (Promega ili Roche), 2 μL DTT (10X) 0,1 M (Promega ili Roche), 6 μL 10 mM ATP, CTP i GTP (10X) 2 μL od svakoga, 1,3 μL 10 mM UTP (10X), 0,7 μL DIG-11-UTP (10X, Roche), ukupni volumen 18 μL. Nakon inkubacije kroz 1 sat na 37 °C, u ovu transkripcijsku smjesu dodan je 1 μL DNA kalupa bez RNaza i 1 μL SP_6, T_3 ili T_7 polimeraze. Neprepisani DNA kalup je uklonjen pomoću 2 μL DNaze (Roche) u 27 μL pufera DNaze (40 mM TRIS, pH = 7,5; 6 mM $MgCl_2$; 10 mM NaCl) i 1 μL inhibitora RNaza kroz 30 min na 37 °C. Reakcija je okončana pomoću 5 μL 0,2 M EDTA, pH = 8. U ovom trenutku je ukupni volumen naše probe bio 55 μL. Konačno pročišćenje RNA probe provedeno je precipitacijom s 1 μL glikogena (20 mg/mL, Roche), 5,6 μL 3 M Na-acetata i 200 μL (prethodno ohlađenog, –20 °C) 100%-tnog etanola na –20 °C preko noći. Probe su centrifugirane 30 minuta na 4 °C. Kuglica je isprana u 1,0 mL ledenog (-20 °C) etanola, centrifugirana 10 minuta na 4 °C i osušena na zraku. Pro-

Materials and methods

Preparing the RNase-free DNA templates

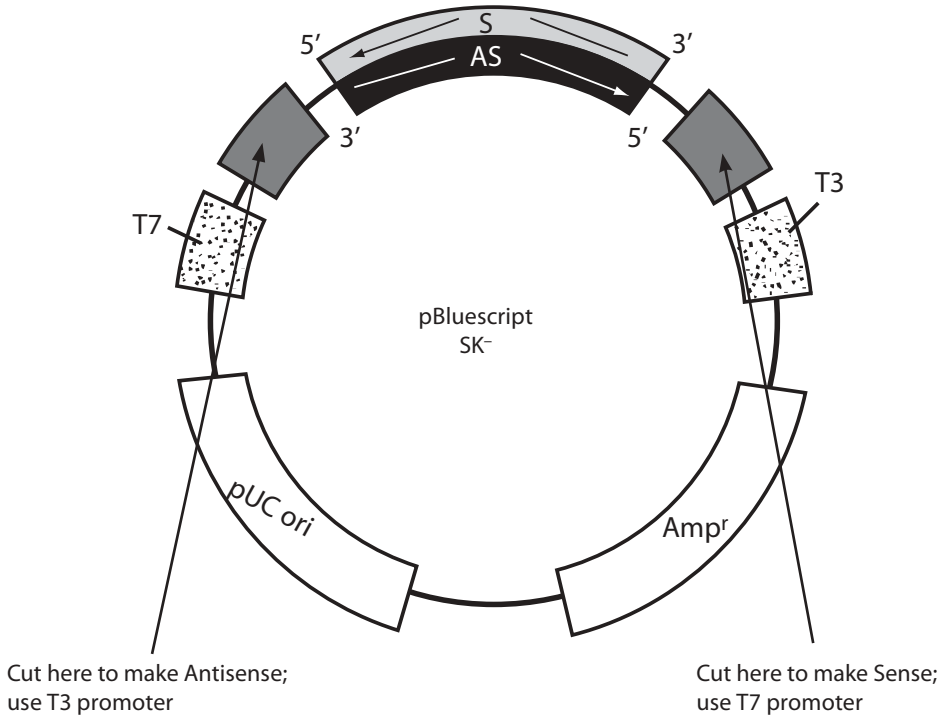
RNA probes for *in situ* hybridization were transcribed with an *in vitro* transcription reaction. Each different cDNA probe construct inserted in a plasmid vector for *in vitro* transcription contained a T_3, T_7 or SP_6 promoter. To transcribe each probe only from the desired cDNA (not from the whole vector) we cut our vectors and made linearized DNA templates.

To prepare RNase-free DNA templates, approximately 20 μg of plasmid construct were digested, containing cDNA for probe, with the desired restriction endonuclease (Fig. 1) using 100 μL reaction volume. This amount was enough to enable labeling at least 10 times.

Linearized templates were extracted with TRIS-HCl saturated phenol, pH = 8 (RNase free) and chloroform isoamyl alcohol (49:1), at a ratio of 1:1, twice, and once with chloroform only. The aqueous phase was precipitated with 3 M sodium acetate, pH = 5.5 (final molarity of 0.3 M sodium acetate) and 3 volumes of 100% ethanol overnight at -20 ˚C. The DNA pellet obtained by centrifugation was washed with ice cold 70% ethanol (diluted with RNase free water from 100% ethanol). The air dried pellet was dissolved in 20 μL 10 mM TRIS (pH = 7.5) (RNase free) and stored at -20 ˚C until later use. The measured concentrations of templates for probes were between 0.5 and 1.5 μg/μL.

Preparing the antisense and sense mRNA probes for *in situ* hybridization

For each probe, we used standard in vitro transcription reaction with digoxigenin UTP (16). Our prepared transcription mix contained: 1 μL RNase inhibitor (40 U/μL), 3 μL RNase free, DEPC (diethyl pyrocarbonate, Sigma) treated H_2O (17), 4 μL 5X SP6 or T3, T7 polymerase buffer (Promega or Roche), 2 μL DTT (10X) 0.1 M (Promega or Roche), 6 μL 10 mM ATP, CTP and GTP (10X) 2 μL of each, 1.3 μL 10 mM UTP (10X), 0.7 μL DIG-11-UTP (10X, Roche), total volume 18 μL. In this transcription mix, 1 μL of RNase free DNA template and 1 μL of SP6, T3 or T7 polymerase were added following incubation at 37 ˚C for 1 hour. The untranscribed DNA template was removed using 2 μL of DNase (Roche) in 27 μL of DNase buffer (40 mM TRIS, pH = 7.5; 6 mM MgCl2; 10 mM NaCl) and 1 μL RNase inhibitor for 30 min at 37 ˚C. The reaction was terminated with 5 μL 0.2 M EDTA, pH = 8. At this point, the total volume of our transcribed probe was 55 μL. Final purification of RNA probe was done with precipitation with 1 μL glycogen (20 mg/mL, Roche), 5.6 μL 3 M Na-acetate and 200 μL (pre-chilled, -20 ˚C) 100% ethanol at -20 ˚C overnight. The probes were centrifuged for 30 minutes at 4 ˚C. The pellet was washed in 1.0 mL of ice cold (-20 ˚C) 70% ethanol, centrifuged for 10 minutes at 4 ˚C and air dried. Probes were dissolved



Slika 1. Ova slika je primjer kako stvoriti *antisense* i *sense* RNA probe transkripcijom cDNA kalupa iz plazmidnog sklopa. Dio plazmidnog sklopa obojen svijetlo sivo (S-obilježeni je *sense* lanac, sekvenca ista kao mRNA) i crno (AS-obilježeni je *antisense* lanac, sekvenca komplementarna mRNA) predstavlja umetak cDNA probe kloniran na poliklonskom mjestu plazmida (obojen tamno sivo). Bluescript plazmid ima polimeraze T3 i T7 (točkasto), uporaba kojih može transkribirati *sense* ili *antisense* RNA probe uz primjenu cDNA umetka kao kalupa probe. Da bismo lokalizirali mRNA u tkivu, stvoriti ćemo *antisense* RNA probu koja je komplementarna mRNA sekvenci (*sense*). *Sense* probe se mogu rabiti za kontrolu. Za stvaranje *antisense* RNA probe plazmid treba prije transkripcije izrezati s odgovarajućim restrikcijskim enzimom blizu 5′ *sense* lanca (svijetlo sivo) cDNA inserta. Izrezivanje plazmida ograničiti će samo transkripciju cDNA inserta, isključujući vektorske sekvence. Nova *antisense* RNA proba može se sintetizirati iz 3′ završetka *sense* cDNA lanca (svijetlo sivo) kao kalup uz uporabu pripadajuće polimeraze (u ovom slučaju T3). Za stvaranje *sense* RNA probe plazmidnu DNA treba izrezati s drugim odgovarajućim restrikcijskim enzimom blizu 3′ završetka *sense* cDNA lanca (svijetlo sivo) i može se sintetizirati iz 3′ završetka *antisense* cDNA lanca (crno) kao kalup uporabom pripadajuće polimeraze (u ovom slučaju T7).

Figure 1. This figure is an example how to generate *antisense* and *sense* RNA probes transcribing cDNA template from a plasmid construct. The portion of the plasmid construct colored light grey (S-labeled is *sense* strand, sequence same as mRNA) and black (AS-labeled is *antisense* strand, sequence complementary to mRNA) represents cDNA probe insert cloned in polyclonal site of plasmid (dark gray). Bluescript plasmid has T3 and T7 polymerases (dots), which utilization can transcribe *sense* or *antisense* RNA probes using cDNA insert as probe template. To localize mRNA in tissue, we would generate an *antisense* RNA probe which is complementary to mRNA sequence (*sense*). *Sense* probes can be used for control. In order to generate an *antisense* RNA probe, the plasmid should be cut with an appropriate restriction enzyme close to the 5′ *sense* strand (light grey) of cDNA insert prior to transcription. Cutting the plasmid will limit cDNA insert transcription only, excluding vector sequences. The new *antisense* RNA probe can be synthesized from the 3′ end of the *sense* cDNA strand (light grey) as template using corresponding polymerase (T3, in this example). To generate a *sense* RNA probe, plasmid DNA should be cut with another appropriate restriction enzyme close to 3′ end of *sense* cDNA strand (light grey) and can be synthesized from 3′ end of the *antisense* cDNA strand (black) as template, using corresponding polymerase (T7, in this example).

be su rastopljene dodatkom 200 μL H_2O bez RNaza, uz 2 μL inhibitora RNaza (kako bi se očuvala RNA) i zagrijane na 37 °C, uz vrtnju nekoliko puta tijekom 20-minutne inkubacije. RNA probe su potvrđene elektroforezom na 5% poliakrilamidnim gelovima koji su sadržavali 15 M ureje, ili Northern analizom, pomoću RNA biljega, uz primjenu 1 μL prepisane probe. Probe su kvantificirane primjenom *DOT blota* za procjenu iskorištenja DIG-obilježene RNA. Probe su pohranjene na –70 °C u alikvotima od po 0,5 ili 1 μg.

by adding 200 μL RNase free H_2O, with 2 μL RNase inhibitor (to preserve RNA) and heated at 37 ˚C, vortexing several times during 20-min incubation. RNA probes were confirmed by electrophoresis on 5% polyacrylamide gels containing 15 M urea, or by Northern analysis, with RNA markers, using 1 μL of transcribed probe. Probes were quantified using *DOT* blot for Estimating the Yield of DIG-labeled RNA. Probes were stored at -70 ˚C in aliquots of 0.5 or 1 μg each.

Probe veće od 1 kb su hidrolizirane radi boljeg prodiranja u tkivo (18). Za hidrolizu RNA probi rabili smo alkalni pufer za hidrolizu bez RNAza 0,2 M (80 mM Na_2HCO_3/120 mM Na_2CO_3), pH = 10,2. Vrijeme inkubacije za hidrolizu izračunali smo prema jednadžbi:

$$T = L_0 - L_f / L_0 \times L_f \times K,$$

gdje je T vrijeme u minutama; L_0 početna duljina probe u kb; L_f željena duljina probe u kb (mi smo rabili 200 bp); K = 0,11 (cijepanje lanca/kg na minutu); primjer:

$$1 \text{ kb} - 0{,}2 \text{ kb} / 1 \times 0{,}2 \times 0{,}11 = 0{,}8 \text{ kb} / 0{,}22 = 36 \text{ min.}$$

Reakcija hidrolize provedena je (prema potrebi) nakon digestije pomoću DNaze prije konačnog pročišćavanja probe precipitacijom etanolom. Primijenili smo jednak volumen obilježene RNA probe (50 μL) i pufera za hidrolizu (50 μL) na 60 °C, prilagođavajući vrijeme izračunavanjem gornje jednadžbe. Reakcija hidrolize je zaustavljena pomoću 1/10 (10 μL) volumena Na-acetata, pH = 5,5 i 1/20 volumena (5 μL) 10% ledene octene kiseline (razrijeđene vodom bez RNAza), nakon precipitacije pomoću 3 volumena (300 μL) 100%-tnog etanola, jednako kako je gore opisano za konačno pročišćavanje RNA probe, prilagođavajući postupak za veće volumene.

Kvantifikacija digoksigeninom obilježenih RNA probi

Prije pohrane na –70 °C RNA probe su kvantificirane pomoću *DOT blota* na pozitivno nabijenoj najlonskoj membrani (Slika 2.). Sve digoksigeninom obilježene probe i kontrolna digoksigeninom obilježena RNA serijski su razrijeđene u omjerima 1:10, 1:100, 1:1000 i 1:10.000. Po 1 μL svakog razrjeđenja ukapan je na najlonsku membranu i fiksiran UV transiluminatorom. Najprije je filtar kratko ispran u Puferu 1 (100 mM TRIS-HCl, 150 mM HCl, pH = 7,5) te 5 minuta u Puferu 2 (pufer 1 + 2% reagens za blokiranje, Roche), a potom obrađen konjugatom anti-digoksigeninskih antitijela i alkalne fosfataze (Roche), razrijeđen u omjeru 1:5000 u Puferu 2. Zatim smo proveli dva ispiranja po 5 minuta u Puferu 1 i dvije minute u Puferu 3 (100 mM TRIS-HCl, 100 mM NaCl, 50 mM $MgCl_2$, pH = 9,5). Za razvijanje boje alkalne fosfataze tijekom 30 minuta na sobnoj temperaturi upotrijebili smo 0,4 mL svježe razrijeđene NBT/BCIP Stock otopine (Roche) ili 90 μL otopine NBT (Roche) i 70 μL BCIP (Roche) u 20 mL pufera 3.
Primjenjujući gore opisani postupak većina naših probi bila je iste koncentracije kao kontrolna proba nakon obilježavanja. Kontrolna proba bila je koncentracije 0,1 μg/mL. Napravili smo alikvote od 10 μL (1 μg) u svakoj epruveti za sve probe. Alikvotirane probe su spremljene na –70 °C kroz godinu dana za buduće pokuse. Za pokus smo svakom alikvotu dodali 1 mL hibridizirane otopine.

The probes larger than 1 kb were hydrolyzed for better penetration into the tissue (18). For hydrolysis of RNA probes, we used RNase free alkaline Hydrolysis Buffer 0.2 M (80 mM Na_2HCO_3/120 mM Na_2CO_3), pH = 10.2. Incubation time for hydrolysis was calculated using the equation:

$$T = L_0 - L_f / L_0 \times L_f \times K,$$

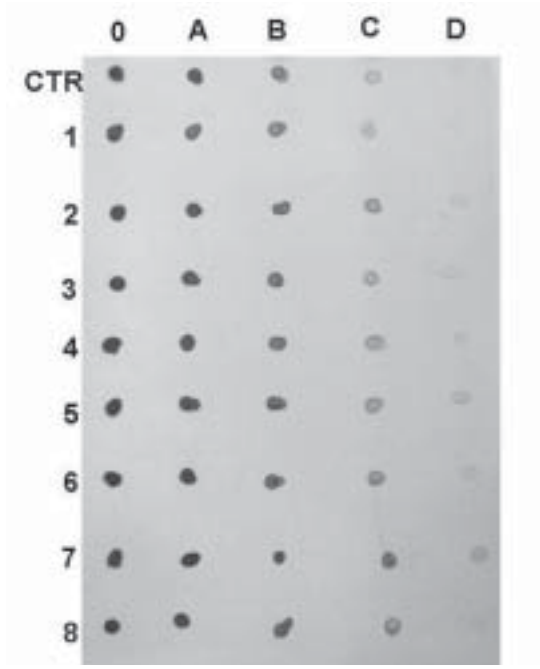
where T is time in minutes; Lo, initial length of probe in kb; Lf, desired length of probe in kb (we used: 200 bp); K = 0.11 (strand scissions/kb *per* minute); example:

$$1 \text{ kb} - 0.2 \text{ kb} / 1 \times 0.2 \times 0.11 = 0.8\text{kb} / 0.022 = 36 \text{ min.}$$

Hydrolysis reaction was performed (if needed) after digestion with DNase and before final purification of probe with ethanol precipitation. We used equal volume of labeled RNA probe (50 μL) and hydrolysis buffer (50 μL) at 60 ˚C adjusting time by calculating the above equation. The hydrolysis reaction was stopped with 1/10 (10 μL) of the volume of Na-acetate pH = 5.5 and 1/20 volume (5 μL) 10% glacial acetic acid (diluted with RNase free water), following precipitation with 3 volumes (300 μL) of 100% ethanol, the same as described above in Final Purification of RNA probe, adjusting procedure for larger volumes.

Quantification of digoxigenin labeled RNA probes

Before storage at -70 ˚C, the RNA probes were quantified using "DOT" blot on a positive charged nylon membrane (Fig. 2). All digoxigenin labeled probes and control digoxigenin labeled RNA were serial diluted to 1:10, 1:100, 1:1,000 and 1:10,000. One μL of each dilution was dropped on the nylon membrane and fixed with a UV transilluminator. First, the filter was washed briefly in Buffer 1 (100mM TRIS-HCl, 150 mM HCl, pH = 7.5), and 5 minutes in Buffer 2 (buffer 1 + 2% blocking reagent, Roche), then treated with anti-digoxigenin antibody-alkaline phosphatase conjugate (Roche), diluted 1:5000 in Buffer 2. Next, we performed two washes for 5 minutes each in Buffer 1 and two minutes in Buffer 3 (100 mMTRIS - HCl, 100 mM NaCl, 50 mM $MgCl_2$, pH = 9.5). For alkaline phosphatase color development, during 30 minutes at room temperature, we used 0.4 mL fresh diluted NBT/BCIP Stock Solution (Roche) or 90 μL NBT Solution (Roche) and 70 μL BCIP (Roche) in 20 mL of buffer 3.
Using the procedure described above, most of our probes were of the same concentration as the control probe after labeling. The control probe was 0.1 μg/mL. We made aliquots of 10 μL (1 μg) in each tube for all probes. The aliquoted probes were stored at -70 ˚C for up to one year for future experiments. For the experiment, we added 1 mL of hybridization solution on each aliquot.



Slika 2. Kvantifikacija digoksigeninom obilježenih probi uz primjenu *DOT blot* i kontrolne obilježene RNA tvrtke Roche. Točke obilježene kao 0 predstavljaju nerazrijeđene probe. **A** predstavlja razrjeđenje probi 1/10, **B** 1/100, **C** 1/1000 i **D** 1/10000. Koncentracija kontrolne probe je 0,1 μg/mL. U ovom primjeru su naše obilježene probe (1-8) imale sličnu koncentraciju kao kontrolna proba (CTR).

Figure 2. Quantification of digoxigenin labeled probes using dot blot and control labeled RNA from Roche. Dots labeled as 0 represent undiluted probes. **A**, represents 1/10; **B**, 1/100; **C**, 1/1000 and **D**, 1/10000 dilution of probes. The concentration of control probe is 0.1 μg/mL. In this example our labeled probes (1-8) have a similar concentration as the control probe (CTR).

Priprava histoloških rezova

Koštano, zubno i meko tkivo je usitnjeno, čuvano na ledu i fiksirano u svježem 4%-tnom paraformaldehidu preko noći na 4 °C (19). Nakon demineralizacije u 15% EDTA, pH = 7,5 i 0,5%-tnom paraformaldehidu kroz 6 tjedana na 4 °C, koštani i zubni uzorci su dehidrirani (uzorci mekog tkiva su dehidrirani slijedećega dana bez demineralizacije) u rastućim koncentracijama metanola (25%, 50%, 80%, 100% dva puta) na ledu, svaka izmjena po 1 sat. Rezovi su uklopljeni u parafin na 56 °C uz 3 izmjene parafina od po 1 sat. Više temperature i duža vremena inkubacije mogu kasnije remetiti imunocitokemiju ako je potrebna nakon *in situ* hibridizacije. Sva vremena u ovom postupku bila su namještena za komadiće tkiva koji nisu veći od 5 mm u promjeru. Za veće komadiće tkiva potrebna su duža vremena u postupku. Sve otopine nabavljene su bez RNAza ili su obrađene pomoću DEPC (17). Za razmazivanje rezova na predmetna stakalca rabili smo vodu bez RNAza (obrađenu pomoću DEPC) koja je prethodno zagrijana na 50 °C, jer parafin koji smo upotrebljavali ima točku taljenja na 52 °C. Upotrebljavali smo prethodno očišćena i obrađena stakalca nabavljena od Fisher Scientific, koja osiguravaju vrlo nisku pozadinu i optimalno prianjanje tkiva: stakalca ProbeOn Microscope ili Superfrost/Plus

Preparation of histologic sections

Bone, tooth and soft tissues were dissected, kept on ice and fixed in fresh 4% paraformaldehyde overnight at 4 °C (19). After demineralization in 15% EDTA pH = 7.5, and 0.5% paraformaldehyde for 6 weeks at 4 °C, bone and tooth samples were dehydrated (soft tissue samples were dehydrated the next day without demineralization) in increasing concentrations of methanol (25%, 50%, 80%, 100%-twice) on ice, each change for 1 hour. The sections were embedded in paraffin at 56 °C with 3 paraffin changes one hour each. Higher temperatures and longer incubation times can interfere later with immunocytochemistry if required after *in situ*. All timings in this procedure were adjusted for tissue pieces not bigger than 5 mm in diameter. For larger tissue pieces, longer times in procedure are required. All solutions used were purchased RNase free, or treated with DEPC (17). To spread the sections for mounting on slides, we used RNase free water (DEPC treated) preheated at 50 °C since the paraffin we used has the melting point at 52 °C. We used pre-cleaned and pretreated slides from Fisher Scientific, which gave us very low background and optimal tissue attachment: ProbeOn Microscope slides or Superfrost/Plus Microscope slides. After mounting, the slides were shaken by

Microscope. Nakon nanošenja pripravke smo ručno protresli kako bi se uklonile kapljice vode između reza i tkiva. Stakalca su ostavljena preko noći na stalku okomito na površinu grijača na 55 °C. Rezovi su spremljeni u kutije na +4 °C s desikatorom, tako da će godinama zadržati signal za *in situ* hibridizaciju i imunocitokemiju. Radi boljeg prianjanja su prije *in situ* hibridizacije stakalca s rezovima kroz noć do slijedećeg jutra položena vodoravno na zagrijanu ploču na 55 °C (to se nije radilo za imunocitokemiju). Rezovi obrađeni dodatnom toplinskom inkubacijom čuvaju mRNA i takvi rezovi su bolje prionuli uza stakalca kroz čitavo vrijeme *in situ* hibridizacije.

Metode 1, 2 i 3; neradioaktivna *in situ* hibridizacija; postupci s primjenom digoksigeninom obilježenim probama

Neradioaktivna *in situ* hibridizacija provedena je postupcima koje smo razvili u našem laboratoriju uz metode koje opisuju drugi istraživači uz primjenu digoksigeninskih probi (7,20). Prije hibridizacije rezovi su deparafinizirani ksilenom i 100%-tnim etanolom nakon rehidracije 50%, 75% i 95% etanolom i PBS (razrijeđenim vodom bez RNAza). Nakon obrade otopinom proteinaze K (Proteinase K, 5 mg/mL u 50 mM TRIS, 5 mM EDTA, pH = 7,6) kroz 10 minuta na 37 °C rezovi su ponovno fiksirani u 4%-tnom formaldehidu u PBS (0,2 M fosfatnog pufera, 0,3 M NaCl), acetilirani (100 mM trietanolamina, 0,25% octeni anhidrid) i predhibridizirani u 2XSSC. Hibridizacija je provedena preko noći na 55 °C u hibridizacijskoj otopini koja je sadržavala 50%-tni formamid, 20 mM TRIS-HCl (pH = 8,0), 1 mM EDTA, 0,3 M NaCl, 10% dekstran sulfat, 1X Denhardtove otopine, 100 μg/mL denaturirane Salmon-Sperm DNA, 500 μg/mL tRNA) i 1 μg/mL DMP1 UTP-digoksigeninom obilježene RNA probe. Nakon hibridizacije su uklonjene pokrovne folije u 5XSSC na sobnoj temperaturi, te su rezovi isprani u 50% formamidu, 5XSSC i 1% SDS otopini na 55 °C dva puta po 30 minuta. Potom su rezovi inkubirani pomoću RNAza (40 mg/mL RNaze A_1 i 10 U/mL RNaze T_1) u otopini RNaza pufera (0,3 M NaCl, 10 mM TRIS, 5 mM EDTA) na 37 °C kroz jedan sat, nakon čega je slijedila inkubacija u istom puferu bez RNAza kroz 30 minuta. Uzastopno ispiranje na 55 °C uslijedilo je dva puta uz 50% formamid i 2XSSC kroz 30 minuta, te svako tri puta po 5 minuta na sobnoj temperaturi u TBST (100 mM TRIS pH = 7,5, 150 mM NaCl, Tween–20 0,1%). Sada su pripravci bili spremni za otkrivanje hibridizacijskog signala trima različitim metodama.

Otkrivanje signala *in situ* hibridizacije

Metoda 1: otkrivanje alkalnom fosfatazom

Nakon ispiranja rezovi su se kroz 1 sat na sobnoj temperaturi namakali u smjesi za blokiranje: 1% reagens za blokiranje (Roche), svježe razrijeđen u puferu maleične kiseline (100 mM maleične kiseline, 150 mM NaCl, kruti NaOH

hand until all water droplets were removed between the section and tissue. Slides were left in a rack perpendicular to surface of heater set at 55 °C overnight. The sections were stored in boxes at +4 °C with desiccant and will retain an *in situ* hybridization and immunocytochemistry signal for years. For better attachment prior to *in situ* hybridization, the night before the slide sections are laid horizontally on a headed plate set at 55 °C until the next morning (for immunocytochemistry this is omitted). Sections treated with the extra heated incubation preserve mRNAs and the sections remained better attached on slides throughout the *in situ* hybridization procedure.

Methods 1, 2 and 3: non-radioactive *in situ* hybridization; procedures using digoxigenin labeled probes

The non-radioactive *in situ* hybridization was performed using procedures developed in our laboratory plus methods from others using digoxigenin probes as described (7,20). Prior to hybridization, sections were de-paraffinized with xylene and 100% ethanol following re-hydration with 50%, 75% and 95% Ethanol and PBS (diluted with RNase free water). After treatment with proteinase K solution (Proteinase K, 5 mg/mL in 50 mM TRIS, 5 mM EDTA pH = 7.6) for 10 minutes at 37 °C, sections were re-fixed in 4% formaldehyde in PBS (0.2 M phosphate buffer, 0.3 M NaCl), acetylated (100 mM triethanolamine, 0.25% acetic anhydride) and pre-hybridized in 2XSSC. Hybridization was performed at 55 °C overnight, in the hybridization solution containing 50% formamide, 20 mM TRIS-HCl (pH = 8.0), 1 mM EDTA, 0.3 M NaCl, 10% Dextran sulfate, 1X Denhardt solution, 100 μg/mL denatured sheared Salmon Sperm-DNA, 500 μg/mL tRNA) and 1 μg/mL DMP1 UTP-digoxigenin labeled RNA probe. After hybridization, the parafilm cover slips were removed in 5XSSC at room temperature and sections were washed in 50% formamide, 5XSSC and 1% SDS solution at 55 °C two times for 30 minutes. Next, the sections were incubated with RNase (40 mg/mL RNase A_1 and 10 U/mL RNase T_1) in RNase buffer solution (0.3 M NaCl, 10 mM TRIS, 5 mM EDTA) at 37 °C for one hour, followed by incubation in the same buffer without RNase for 30 minutes. Consecutive washes at 55 °C were done twice with 50% formamide and 2XSSC for 30 minutes, and three times for 5 minutes each at room temperature in TBST (100 mM TRIS pH = 7.5, 150 mM NaCl, Tween-20 0.1%). At this point, slides were ready for detection of hybridization signal with three different methods:

Detection of *in situ* hybridization signal

Method 1: Alkaline phosphatase detection

After washing, the sections were soaked for 1 hour at room temperature in blocking mix: 1% blocking reagent- (Roche), fresh diluted in maleic acid buffer (100 mM Ma-

ili 10 N kako bi se namjestio pH = 7,5). Odsječci anti-Digoxigenin-AP-Fab (Boehringer Mannheim ili Roche) razrijeđeni su u omjeru 1:50 za probu koštanog morfogenetičnog proteina 2 (BMP2), osteokalcina (OC) i osteopontina (OPN), 1:500 u smjesi za blokiranje za probu kolagena 1a1 (Col1) i dodani nakon poslijehibridizacijskog ispiranja i inkubiranja za inkubaciju preko noći na 4 °C. Slijedećega dana su rezovi isprani pomoću TBST kroz 5 minuta puferom za ispiranje (100 mM maleične kiseline, 150 mM NaCl, Tween–20 0,1%, 2 mM levamisola), dva puta kroz 15 minuta, te preddetekcijskim puferom (100 mM TRIS pH = 9, 100 mM NaCl, 2 mM levamisola) kroz 5 minuta. Otkrivanje hibridizacijskog signala provedeno je prema De Blocku i sur. (21) dodavanjem supstrata alkalne fosfataze (NTB/BCIP, Boehringer Mannheim ili Roche) ili NTB (Boehringer Mannheim ili Roche) i BICP (Roche u pufer za detekciju (10% polivinil alkohol 70–100 kd-Sigma, 100 mM TRIS pH = 9, 100 mM NaCl, 2 mM, 50 mM $MgCl_2$, 2 mM levamisola) kako bi se inhibirala endogena alkalna fosfataza, te 10 mM N-etil maleimida za inhibiranje reakcije „dehidrogenaze ničega" (22). Trajanje razvoja hibridizacijskog signala bilo je od 1 sata do preko noći na 30 °C, ovisno o probi. Kad je otkrivanje hibridizacijskog signala završeno, rezovi su isprani toplom vodom 3 puta po 5 minuta (može se ostaviti u vodi nekoliko dana na +4 °C) i kontraobojeni metilnim zelenim ili nuklearnim brzim crvenilom kroz 5 minuta, dehidrirani u etanolu, isprani u ksilenu i naneseni.

Metoda 2: umnožavanje signala tiramidom i otkrivanje alkalnom fosfatazom

In situ hibridizacija provedena je na isti način kao kod 1. metode, jedino je dodano prigušivanje faze endogene peroksidaze (3% H_2O_2 u PBS kroz 60 minuta) prije digestije pomoću proteinaze K, nakon toga 3 5-minutna ispiranja pomoću PBS. Taj je korak bio neophodan kako bi se pozadina svela na najmanju mjeru, jer hrenova peroksidaza (HRP) katalizira aktiviranje i kovalentno vezanje DNP amplifikacijskog reagensa.

Za ovo otkrivanje rabili smo *in situ* sustav TSA plusDNP-AP, Perkin Elmer. Nakon poslijehibridizacijskog ispiranja rezovi su namočeni kroz 30 minuta na sobnoj temperaturi u TNB reagensu za blokiranje: 0,5% reagensa za blokiranje-Perkin Elmer (ili je bila priložena u setu), svježe pripravljena ili pohranjena na –20 °C u TNT puferu (100 mM TRIS pH = 7,5; 150 mM NaCl; 0,05% Tween–20). Antitijelo Anti Digoxigenin-POD (Poly) Fab odsječaka (Boehringer Mannheim ili Roche) razrijeđeno je u omjeru 1:50 u reagensu za blokiranje i inkubirano preko noći na 4 °C nakon 3 puta po 5 minuta ispiranja u TNT-u. Slijedeći korak je bila 3- do 10-minutna inkubacija u reagensu za umnožavanje signala tiramidom (razrjeđena u omjeru 1:50 u 1 x amplifikacijskom razrjeđivaču, oboje isporučeno u setu), nakon 3 puta po 5 minutnih ispiranja u TNT. Nakon ovih ispiranja

leic Acid, 150 mM NaCl, NaOH solid or 10N to adjust pH = 7.5). Anti-Digoxigenin-AP-Fab fragments (Boehringer Mannheim or Roche) were diluted for bone morphogenetic protein 2 (BMP2), osteocalcin (OC) and osteopontin (OPN) probe 1:50, for collagen 1a1 (Col1) probe 1:500 in blocking mix and was added after post-hybridization washing and incubating for overnight incubation at 4 °C. Next day, the sections were washed using TBST for 5 minutes, wash buffer (100 mM Maleic acid, 150 mM NaCl, Tween-20 0.1%, 2 mM Levamisole) twice for 15 minutes, and pre-detection buffer (100 mM TRIS pH = 9, 100 mM NaCl, 2 mM Levamisole) for 5 minutes. Detection of hybridization signal was done according to De Block *et al.* (21) by adding alkaline phosphatase substrate (NTB/BCIP, Boehringer Mannheim or Roche) or NTB (Boehringer Mannheim or Roche) and BICP (Roche) in detection buffer (10% polyvinyl alcohol 70-100 kd-Sigma, 100 mM TRIS pH = 9, 100 mM NaCl, 2 mM, 50 mM $MgCl_2$, 2 mM Levamisole), to inhibit endogenous alkaline phosphatase, and 10 mM N-ethyl maleimide to inhibit "nothing dehydrogenase" reaction (22). The duration of hybridization signal development was from 1 hour to overnight at 30 °C, depending on the probe. When detection of hybridization signal was completed the sections were washed in warm water 3 x 5 minutes (can be left in water for few days at +4 °C) and counterstained with methyl green or nuclear fast red for 5 minutes, dehydrated in ethanol, washed in xylene and permounted.

Method 2: Tyramide signal amplification and alkaline phosphatase detection

In situ hybridization was done as for method 1, only quenching of endogenous peroxidase step (3% H_2O_2 in PBS for 60 minutes) was added before proteinase K digestion followed by three 3x5 minutes washes with PBS. This step was necessary to minimize background, since activation and covalent binding of DNP Amplification reagent is catalyzed by horseradish peroxidase (HRP).

For this detection we used *in situ* TSA plusDNP-AP system from PerkinElmer. After post hybridization washing, the sections were soaked for 30 minutes at room temperature in TNB blocking reagent: 0.5% blocking reagent-PerkinElmer (or provided in kit), fresh made or stored at -20 °C in TNT buffer (100 mM TRIS pH = 7.5; 150 mM NaCl; 0.05% Tween-20). Anti Digoxigenin-POD (Poly) Fab fragments antibody (Boehringer Mannheim or Roche) was diluted 1:50 in blocking reagent, and incubated overnight at 4 °C following 3 x 5 minute washes in TNT. Next step was 3- to 10-min incubation in Tyramide Signal Amplification reagent (dilution from stock 1:50 in 1x Amplification Diluent, both supplied in kit), following 3 x 5 minute washes in TNT. After these washes, the sections were incubated in pre-detection buffer (100 mM TRIS pH = 9, 100 mM NaCl, 2 mM Levamisole) for 5 minutes. Detection of

rezovi su inkubirani u preddetekcijskom puferu (100 mM TRIS pH = 9, 100 mM NaCl, 2 mM levamisola) kroz 5 minuta. Otkrivanje hibridizacijskog signala provedeno je kao kod 1. metode. Trajanje razvoja hibridizacijskog signala bilo je od 10 minuta do 1 sata na 30 °C, ovisno o probi. Rezovi su kontraobojeni i naneseni kao kod 1. metode.

Metoda 3: Umnožavanje signala tiramidom i fluorescentno otkrivanje cijaninom 5

In situ hibridizacija je provedena jednako kao kod 1. metode, samo je dodano prigušivanje faze endogene peroksidaze prije digestije proteinazom K, nakon toga su slijedila tri 3-minutna ispiranja pomoću PBS, kako je opisano za 2. metodu.

U ovoj metodi otkrivanja rabili smo *in situ* sustav TSA PLUS Fluorescence, Perkin Elmer. Nakon poslijehibridizacijskog ispiranja, kao kod 2. metode, rezovi su namočeni kroz 30 minuta na sobnoj temperaturi u TNB reagensu za blokiranje. Antitijelo Anti Digoxigenin-POD razrijeđeno je u omjeru 1:50 u reagensu za blokiranje i inkubirano preko noći na 4 °C nakon 3 5-minutnih ispiranja u TNT. Nakon inkubacije kroz 3–10 minuta u reagensu za umnožavanje signala tiramidom (isporučeno u setu) slijedila su 3 5-minutna ispiranja u TNT. Nakon ovih ispiranja rezovi su naneseni u fluorescentni medij Vector shield i promatrani pomoću konfokusnog mikroskopa (ekscitacija na 648 nm, emisija na 570 nm).

Kvantifikacija hibridizacijskog signala pomoću programa ImageJ

ImageJ je na Javi baziran program za obradu snimaka za javnu uporabu, koji je izradio National Institute of Health (NIH), SAD. Program ImageJ je dostupan za Microsoft Windows, Mac OS, Mac OS X, Linux, Sharp Zaurus PDA i može se prebaciti na čvrsti disk nakon prijave na: http//: rsb.info.nih.gov/ij/download.html.

Ovaj program je izrađen s otvorenom arhitekturom, tako da osigurava mogućnost proširenja putem verzije 1.1 ili kasnijih verzija Java pomoću nadogradnje i makroa koji se mogu snimiti. Prilagođeno prikupljanje, analiza i obrada nadogradnje mogući su pomoću programa za uređivanje ugrađenog u ImageJ i Java prevodnika. Korisnička nadogradnja omogućava rješavanje mnogih problema u obradi i analizi snimaka, od trodimenzijskog prikaza živih stanica do obrade radioloških snimaka, usporedbe podataka višestrukih sustava prikazivanja i automatskih hematoloških sustava.

Mi smo za kvantificiranje signala *in situ* hibridizacije rabili purpurno plavu boju iz NBT formazana, proizvoda stvorenog reakcijom alkalne fosfataze uz pomoć supstrata BCIP i NTB. Kontrabojanje treba biti vrlo lagano kako ne bi remetilo signal mRNA. Prije upotrebe programa ImageJ može se pod *Analyze* u *Set Measurements* izabrati područje, minimalno i maksimalno sivo područje, te srednja

hybridization signal was done as in method 1. Duration of hybridization signal development was 10 minutes to 1 hour at 30 °C, depending on the probe. Sections were counterstained and permounted as in method 1.

Method 3: Tyramide signal amplification and cyanine 5 fluorescence detection

In situ hybridization was done the same as for method 1, only quenching of endogenous peroxidase step was added before proteinase K digestion followed by three 3 minute washes with PBS, as described in method 2.

For this detection, we used *in situ* TSA PLUS Fluorescence system from PerkinElmer. After post hybridization washing, the same as in method 2, the sections were blocked for 30 minutes at room temperature in TNB blocking reagent. Anti Digoxigenin-POD antibody was diluted 1:50 in blocking reagent, and incubated overnight at 4 °C following 3 X 5 minute washes in TNT. Incubation for 3-10 minutes in Tyramide Signal Amplification reagent (supplied in kit) was followed by 3 x 5 minute washes in TNT. After these washes, the sections were permounted in Vector shield fluorescent mounting media and viewed with a confocal microscope (Excitation 648nm, Emission 570 nm).

Quantification of hybridization signal with ImageJ

ImageJ is a public domain, a Java based image processing program developed at the National Institutes of Health (NIH), USA. The ImageJ software is freely available for Microsoft Windows, Mac OS, Mac OS X, Linux, Sharp Zaurus PDA and can be downloaded if logged on: http://rsb.info.nih.gov/ij/download.html.

This software has been designed with an open architecture that provides extensibility *via* Java 1.1 version or later versions, using plugins and recordable macros. Custom acquisition, analysis and processing plugins can be developed using ImageJ's built-in editor and a Java compiler. User-written plugins make it possible to solve many image processing and analysis problems, from 3-dimensional live-cell imaging to radiological image processing, multiple imaging system data comparisons to automated hematology systems.

For quantifying *in situ* hybridization signal, we used a purple-blue color from NBT formazan, which is a generated product of alkaline phosphatase reaction using BCIP and NTB substrate. The counterstain should be very light in order not to interfere with the mRNA signal. Before using ImageJ, the area, minimum and maximum grey area, and mean grey value can be selected in Set Measurements under Analyze. We used the tool "free hand selections" surrounding areas for measurements. All measurements were read clicking on Measure under Analyze. All areas measured for a hybridization signal were corrected for background reading. To define background, 5 areas

vrijednost sivog. Mi smo rabili radnju *„free hand selection"* oko područja mjerenja. Sva mjerenja se očitavaju pritiskom na *Measure* pod *Analyze*. Sva područja mjerena za hibridizacijski signal ispravljena su za očitanje pozadine. Za definiranje pozadine odabrano je i uprosječeno 5 područja bez hibridizacijskog signala, a potom oduzeto od vrijednosti signala. Svi dobiveni brojevi su relativni.

4. metoda: radioaktivna in situ hibridizacija i otkrivanje signala

Radioaktivna *in situ* hibridizacija uvelike se provodila pomoću postupaka razrađenih u našem laboratoriju i opisanih u radu Gluhak-Heinrich i suradnika (23). Hibridizacija je provedena pomoću RNA probi ^{32}P rUTP obilježenog kolagena1a1 (Col1) i osteokalcina (OC).

Rezultati

Cilj ove studije bio je razviti i odrediti snagu naših neradioaktivnih *in situ* tehnika u usporedbi s našom dobro utvrđenom i široko primjenjivanom radioaktivnom metodom (23) uz uporabu parafinskih rezova. Kao nov pristup u dokazivanju korisnosti naših novo razvijenih metoda u različitim primjenama, poglavito s parafinskim rezovima, pokazali smo i mogućnost kvantificiranja signala neradioaktivne hibridizacije. Za ispitivanje ove mogućnosti rabili smo demineralizirane tkivne rezove s koštanim, zubnim i mekim tkivom. Najprije smo razradili i optimirali tri različita postupka za metode neradioaktivne *in situ* hibridizacije. Zatim smo analizirali izraženost Col1 i OC mRNA u rezovima mišje alveolarne kosti blizu prvih kutnjaka pomoću naše rutinske radioaktivne *in situ* analize (slika 3.). Potom smo utvrdili izraženost Col1, OC i osteoponinske (OP) mRNA neradioaktivnim pristupom (slika 4.). Rabili smo točno iste kalupe naših cDNA za prepisivanje RNA probi obilježenih ^{32}P ili digoksigeninom. Izraženost Col1 i OC mRNA u alveolarnoj kosti i zubima usporedili smo i kvantificirali. Kako bismo dokazali da je naša neradioaktivna metoda broj 2 *in situ* hibridizacije jednako osjetljiva kao i naša radioaktivna metoda u mekom i koštanom tkivu, ispitali smo tri različite neradioaktivne metode i radioaktivnu metodu trima različitim probama: BMP2 (vrlo nizak broj kopija mRNA), Col1 (vrlo visok broj kopija mRNA) i OP (srednji broj kopija i specifična za osteocite).

U razvoju naših *in situ* postupaka najprije smo ispitali različite parametre za optimalan rad. Rezultati ovih ispitivanja opisani su niže u tekstu.

Naše smo ispitivanje započeli optimalnom pripravom koštanog tkiva. To je ključno, jer *in situ* hibridizacija uvijek započinje fiksacijom tkiva ili stanica kako bi se očuvala mRNA unutar stanica, kao i tkivna morfologija. Fiksacija paraformaldehidom uz obradu pomoću EDTA treba od početka biti bez RNAza. Isto tako smo utvrdili kako su rezovi debljine 8 mikrona optimalni za naše metode; mogu

without hybridization signal were selected and averaged, then subtracted from the signal value. All obtained numbers are relative.

Method 4: Radioactive in situ hybridization and signal detection

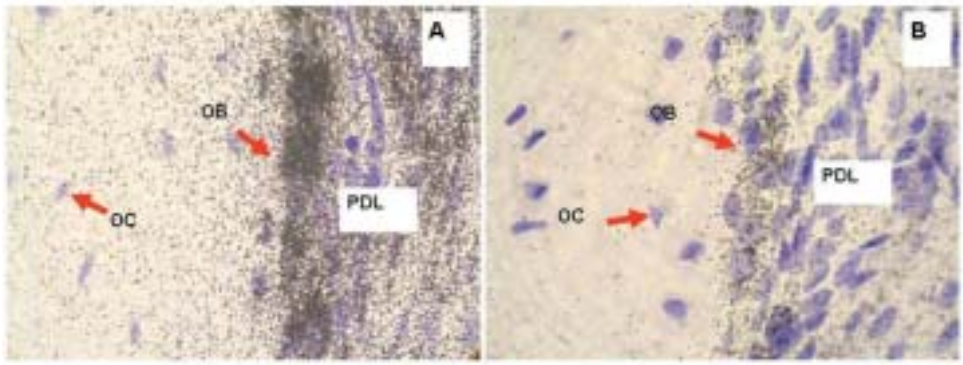
The radioactive *in situ* hybridization was extensively performed using procedures developed in our laboratory and described by Gluhak-Heinrich *et al.* (23). Hybridization was performed with ^{32}P rUTP labeled collagen 1a1 (Col1) and Osteocalcin (OC) RNA probes.

Results

The aim of this study was to develop and determine the power of our non-radioactive *in situ* techniques in comparison to our well established and widely used radioactive method (23) using paraffin sections. As a novel approach to prove usefulness of our newly developed methods in a variety of applications, especially paraffin sections, we also showed the possibility of quantification of non-radioactive hybridization signals. To test this, we used de-mineralized tissue sections with bone, tooth and soft tissues. First we developed and optimized three different procedures of non-radioactive *in situ* hybridization methods. Second, we analyzed the expression of Col1 and OC mRNA in sections of mouse alveolar bone adjacent to first molars using our routine radioactive *in situ* analysis (Figure 3). Third, we determined the expression of Col1, OC and Osteopontin (OP) mRNA using a non-radioactive approach (Figure 4). We used exactly the same templates of our cDNAs to transcribe ^{32}P or digoxigenin labeled RNA probes. The Col1 and OC mRNA expression in alveolar bone and tooth were compared and quantified. To prove that our non-radioactive *in situ* hybridization method 2 is as sensitive as our radioactive method in soft and bone tissue, we tested three different non-radioactive methods and radioactive method with three different probes: BMP2 (very low mRNA copy number), Col1 (very high mRNA copy number) and OP (medium copy number and specific for osteocytes).

In developing our *in situ* procedures, we first tested different parameters for optimal performance. The results of this testing are described below, but *in situ* pictures of these numerous testing are not shown because a large space would be needed.

We started our testing with optimum preparation of bone tissue. This is crucial since *in situ* hybridization always begins with fixation of the tissue or cells, which should preserve mRNA within the cells and morphology of tissue. The paraformaldehyde fixation with EDTA treatment should be RNase free from the beginning. We also found that 8-micron thick sections were optimal for our methods: thicker sections can be used and thickness may give



Slika 3. Radioaktivna *in situ* hibridizacija Collagen1a1 (A) i Osteocalcin (B) u reprezentativnim rezovima mišje maksile. Pozitivan hibridizacijski signal predstavljen je srebrnim zrncima-crnim točkicama na tkivu. Grimizna boja je kontrabojanje hematoksilinom. Signal iznad pozadine je najviši u osteoblastima i za probu Collagen1a1 i za probu Osteocalcin. Vidi se kako je hibridizacijski signal obilniji za kolagen nego za osteokalcin. PD, periodont; OB, osteoblasti; OC, osteociti.

Figure 3. Radioactive in situ hybridization of Collagen 1a1 (A) and Osteocalcin (B) in representative sections of mouse maxilla. Positive hybridization signal is represented with silver grains, black dots over tissue. Purple color is counterstained using hematoxylin. The signal above the background is the highest in osteoblasts for both Collagen 1a1 and Osteocalcin probes. Note that the hybridization signal is more abundant for collagen than for osteocalcin. PD, periodontium; OB, osteoblasts; OC, osteocytes.

se rabiti deblji rezovi i debljina bi nam mogla dati više hibridizacijskog signala, ali će se rezolucija smanjiti ako se snimke rade uz veliko uvećanje i možda ne ostanu prionuti uza stakalca kroz čitavo vrijeme hibridizacijskog postupka.

Najbolji rezultati za linearizaciju DNA probi restrikcijskim endonukleazama dobiveni su enzimskim cijepanjem kojim su proizvedeni 5' ljepljivi ili tupi završetci. Rezultati su također bili bolji ako su probe prije obilježavanja digoksigeninom i prije alikvotiranja radi čuvanja podvrgnute vorteksiranju i zagrijane uz 10 mM TRIS, pH = 7,6 bez RNAza na 65 °C kroz 20 minuta i/ili uz dodatak SDS u 0,1% kako bi se pripomogla topljivost probi.

Dobili smo optimalni signal i nisku pozadinu s 5 μL probe na 1 cm^2 stakalca za hibridizaciju, sa svim digoksigeninom obilježenim probama u koncentraciji od 1 μg na 1 mL hibridizacijske otopine. Uporaba veće količine otopine s više probe na mm^2 na stakalcu ili više koncentrirane probe dala je jaču pozadinu. Utvrdili smo kako se koncentracija probe može točno procijeniti gel elektroforezom i bojanjem etidijevim bromidom uspoređujući alikvote RNA DIG obilježene probe sa standardnom RNA poznate koncentracije (jedna takva se isporučuje sa setom za obilježavanje DIG) ili usporedbom *DOT blota* serije razrjeđenja obilježene RNA probe i obilježenih RNA standarda (Slika 2.).

Za radioaktivnu hibridizaciju procijenili smo količinu probe upotrebljenu u gel elektroforezi i kod bojanja etidijevim bromidom s standardom RNA poznate koncentracije. Koncentracija probi koju smo mi rabili kretala se od 100 do 500 ng/mL hibridizacijske otopine, a na stakalca smo

us more hybridization signal, but resolution will decrease if pictures are taken on high magnification and they may not stay adhered to the slides throughout the hybridization procedure.

Best results for linearizing DNA probes with restriction endonucleases were obtained with enzymes cleaving producing 5' overhangs or blunt ends. The results were also better if probes, after digoxigenin labeling and before aliquoted for storage, were vortexed and heated in RNase free 10 mM TRIS pH = 7.6 at 65 ˚C for 20 minutes and/or the addition of SDS to 0.1% to aid solubility of probes.

We obtained optimal signal and low background with 5 μl of probe *per* 1 cm^2 of slide used for hybridization, with all digoxigenin labeled probes at a concentration of 1 μg in one mL of hybridization solution. Use of more solution with more probe *per* mm^2 on the slide or more concentrated probe gave us higher background. We found that the accurate probe concentration estimation can be obtained by gel electrophoresis and ethidium bromide staining comparing aliquots of the DIG labeled probe RNA with a standard RNA of known concentration (one is supplied with the kit for DIG labeling) or by comparing dot blots of a dilution series of labeled RNA probe and labeled RNA standards (Fig. 2).

For radioactive hybridization, we estimated the amount of probe used by gel electrophoresis and ethidium bromide staining with an RNA standard of known concentration. The concentration of probes we used was 100-500 ng/mL of hybridization solution and on slides we added 80 μl (10 μl *per* 1 cm^2). Regarding probe length, our experiments showed that probes of up to 1 kb were not a

dodali 80 μL (10 μL na 1 cm^2). Glede duljine probe, naši pokusi su pokazali kako sa probama do 1 kb nema problema kod prodiranja u tkivo kod neradioaktivnog *in situ* postupka, ali su za radioaktivni *in situ* postupak sve probe hidrolizirane do prosječne duljine od 150–250 bp kako bi se dobio optimalan signal.

Pri ispitivanju tkivne propustljivosti naša neradioaktivna metoda pokazala je dobre rezultate s 5 μg/mL proteinaze K. Ova je koncentracija prethodno ispitana za našu dobro utvrđenu radioaktivnu metodu (23). Nakon pokusa s različitim koncentracijama proteinaze K najbolje rezultate smo polučili primjenom iste koncentracije kakvu smo rabili za radioaktvni *in situ* postupak, jer su koncentracije veće od 10 μg/mL proteinaze K uništavale morfologiju i povećavale pozadinu.

U našim prijašnjim i usporednim studijama radeći s radioaktivnom *in situ* hibridizacijom analizirali smo digestiju s RNazom tijekom faza ispiranja i njezin učinak na pozadinu. Našli smo da je optimalna koncentracija RNaze 40 mg/mL. Ova nam je količina dala najnižu pozadinu, a veće količine su imale manji utjecaj na pozadinu. Velika razlika u smanjenju pozadine kod *in situ* hibridizacije nastupila je dodavanjem RNaze T_1 u količini do 10 U/mL. Ovo ispitivanje je obavljeno uz radioaktivni *in situ* postupak te je preuzeto za neradioaktivni postupak, pokazujući vrlo čistu, nisku pozadinu.

Ispitivali smo različita razrjeđenja anti-digoksigeninskih antitijela. Najbolje rezultate za visoko zastupljene gene dobili smo s razrjeđenjem 1:500, no za probe koje smo rabili za otkrivanje malobrojnih mRNA ciljeva optimalno razrjeđenje je bilo 1:50.

Temeljito smo ispitivali duljinu vremena za razvijanje obojene otopine enzimatskom reakcijom alkalne fosfataze. Utvrdili smo kako je vrijeme razvijanja za probu Col1 jedan sat, probama OC i OP trebalo je 6 sati, dok je za probu BMP2 kao ciljnu mRNA, koja je prisutna u vrlo niskoj količini, trebalo prekonoćno razvijanje pomoću 1. metode. Ovi su uvjeti vrijedili za razrjeđenje anti-DIG antitijela 1:50. Što su antitijela više razrijeđena, to je više vremena potrebno za razvijanje. Pomoću 2. metode sve su se probe razvile u vremenu od 10 minuta do 1 sata, jer je signal bio pojačan (ovi rezultati nisu prikazani). Naši su rezultati pokazali kako razvijanje duže od 20 sati obično dovodi do jače pozadine nego što je prihvatljivo.

Kvantifikacija rezultata *in situ* hibridizacije dobivenih pomoću Col1 i OC

Kako je prikazano na slikama 4. i 5., vidljivost signala neradioaktivne hibridizacije može se otkriti na različitim razinama. Najviši intenzitet hibridizacijskog signala otkriven je u odontoblastima (OD) pomoću probe Col1 (slika 5A.). Najniži signal je otkriven u osteoblastima (OB) pomoću probe Col1 (slika 5B. i 6B.). Nakon kvantifikacije je hibridizacijski signal najvišeg intenziteta izmjeren pomoću

problem for penetration in the tissue with non-radioactive *in situ*, but for radioactive *in situ* all probes were hydrolyzed to average length of 150-250 bp to obtain optimal signal.

On testing for tissue permeability, our non-radioactive method showed good results with: 5 μg/mL of Proteinase K. This concentration was previously tested for our well established radioactive method (23). After experiments with different concentrations of Proteinase K, we obtained best results using the same concentration as we used for radioactive *in situ*, since concentrations higher than 10 μg/mL of Proteinase K destroyed morphology and increased background.

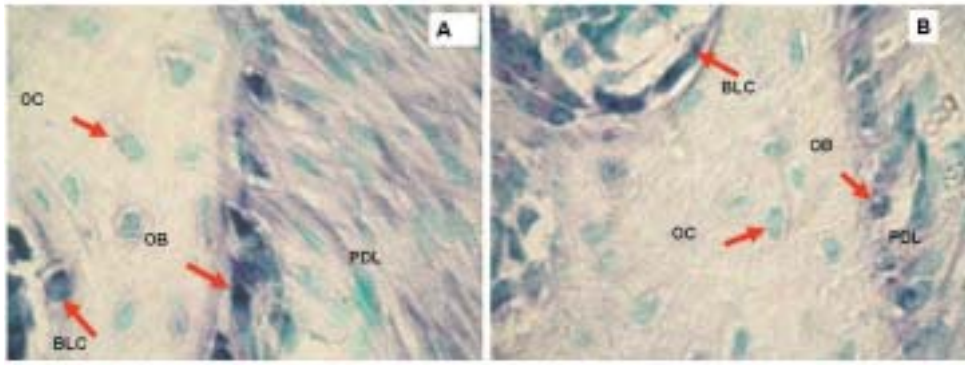
In our previous and parallel studies working with radioactive *in situ* hybridization, we analyzed digestion with RNase during washing steps and its effect on background. We found that 40 mg/mL RNase A to be optimum. This amount gave us lowest background and higher amounts had little influence on background. The great difference in decreasing background *in situ* hybridization was the addition of RNase T_1 up to 10 U/mL. This testing was done with radioactive *in situ* and it was adopted for non-radioactive showing very clear low background.

We tested different dilutions of anti-digoxigenin antibodies. The best results for high abundant genes were at 1:500 dilution but for probes used for detection of few mRNA targets the optimal dilution was 1:50.

The length of time with developing color solution with alkaline phosphatase enzymatic reaction was tested extensively. We found developing time for Col1 probe to be one hour, OC and OP probes needed 6 hours, and for BMP2 probe as a very low abundant mRNA target we needed overnight development using method 1. These conditions are for dilution of anti-DIG-Antibodies 1:50. The more diluted antibodies, the longer the time of development needed. Using method 2, all probes developed between 10 minutes to 1 hour, since the signal was amplified (results not shown). Our results showed that development longer than 20 hours usually resulted in higher background than acceptable.

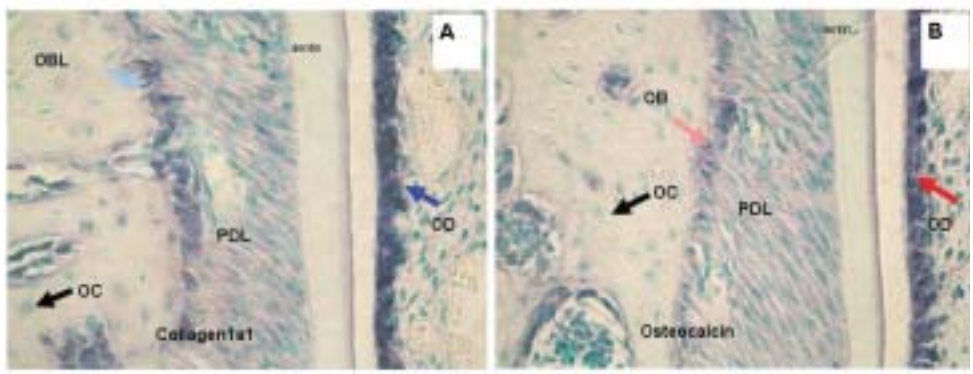
Quantification of *in situ* hybridization results obtained with Col1 and OC

As shown in Figures 4 and 5, visibility of non-radioactive hybridization signal is detectable at different levels. The highest intensity of hybridization signal was detected in odontoblasts (OD) with Col1 probe (Fig. 5A). The lowest signal was detected in osteoblasts (OB) with Col1 probe (Figs. 5B and 6B). After quantification, the hybridization signal of highest intensity was measured with ImageJ as 95% (100% is black) and the lowest detected in this experiment was 23% (0% is white). The other two middle expression levels are: signal in odontoblasts (OD) obtained with OC (80%) and signal in osteoblasts (OB) from Col1



Slika 4. Neradioaktivna *in situ* hibridizacija provedena pomoću digoksigeninom obilježenih probi, Collagen1a1 (A) i Osteocalcin (B) u reprezentativnim rezovima mišje maksile. Pozitivan hibridizacijski signal predstavljen je plavim obojenjem. Zelena boja predstavlja metil zeleno obojenje kao kontraboju. Signal je najviši u osteoblastima i za probu Collagen1a1 i probu Osteocalcin, no isto tako postoji stanovit signal u PDL i stanicama koštane obloge. U ovim rezovima osteociti ne izražavaju niti kolagen niti osteokalcin. Opaža se kako je hibridizacijski signal obilniji za kolagen nego za osteokalcin. PD, periodont; OB, osteoblasti; OC, osteociti; BLC, stanice koštane obloge.

Figure 4. Non-radioactive in situ hybridization performed with digoxigenin labeled probes, Collagen 1a1 (A) and Osteocalcin (B) in representative sections of mouse maxilla. Positive hybridization signal is represented with blue stain. Green color represents methyl green staining as counterstain. The signal is highest in osteoblasts for both Collagen 1a1 and Osteocalcin probes but there is also some signal in PDL and bone lining cells. Osteocytes are not expressing collagen or osteocalcin in these sections. Note also that the hybridization signal is more abundant for collagen than for osteocalcin. PD, periodontium; OB, osteoblasts; OC, osteocytes, BLC, bone lining cells.

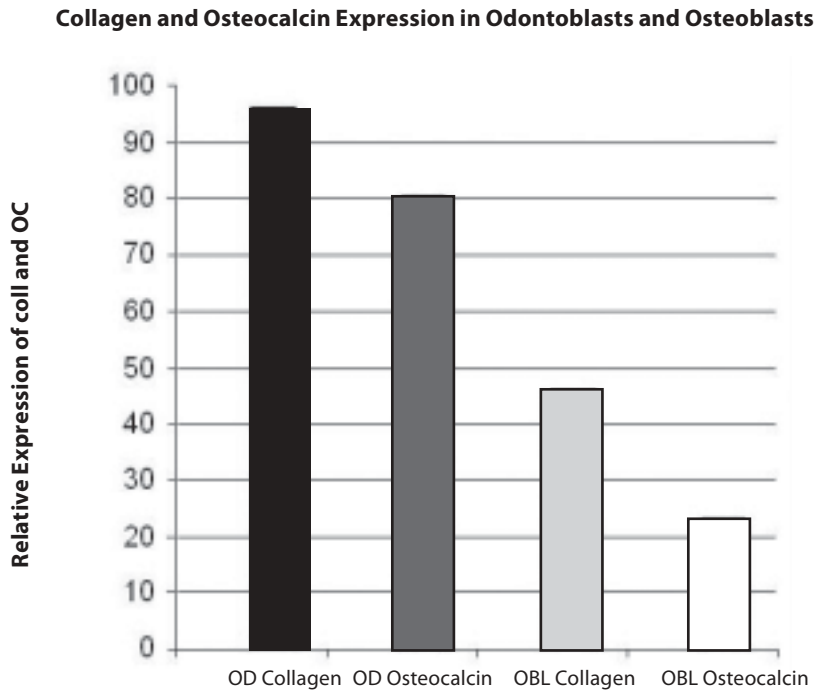


Slika 5. Različita jačina hibridizacijskog signala dobivenog neradioaktivnom digoksigeninom obilježenom *in situ* hibridizacijom Collagen1a1 (A) i Osteocalcin (B) u reprezentativnim rezovima mišje maksilarne alveolarne kosti i dijela prvog kutnjaka. Plavo obojenje predstavlja pozitivan hibridizacijski signal, a zeleno predstavlja kontraboju. Signal je najviši u odontoblastima obilježenima Collagen1a1, nakon čega slijedi signal nižega intenziteta ali još uvijek visok u osteokalcinom obilježenim odontoblastima. Srednja jačina hibridizacijskog signala nalazi se u osteoblastima hibridiziranima pomoću Collagen1a1. Dobro vidljiv ali najniži intenzitet hibridizacijskog signala otkriven je u osteoblastima hibridiziranim osteokalcinskom probom. Stanice koštane obloge također su visoko obilježene. Osteociti ne izražavaju niti kolagen niti osteokalcin u ovim rezovima. Vidi se također kako je hibridizacijski signal obilniji za kolagen nego za osteokalcin i u odontoblastima i u osteoblastima. PD, periodont; OB, osteoblasti; OC, osteociti; BLC, stanice koštane obloge; OD, odontoblasti.

Figure 5. Different intensity of hybridization signal obtained with non-radioactive digoxigenin labeled in situ hybridization of Collagen 1a1 (A) and Osteocalcin (B) in representative sections of mouse maxillary alveolar bone and part of the first molar. The blue stain represents positive hybridization signal and green represents counterstain. The signal is highest in Collagen 1a1 labeled odontoblasts followed by lower intensity but still very high signal in osteocalcin labeled odontoblasts. The middle intensity of hybridization signal is in osteoblasts hybridized with Collagen 1a1. Well visible but the lowest hybridization signal intensity was detected in osteoblasts hybridized with the osteocalcin probe. The bone lining cells are also highly labeled. Osteocytes are not expressing collagen or osteocalcin in these sections. Note also that the hybridization signal is more abundant for collagen than for osteocalcin in both odontoblasts and osteoblasts. PD, periodontium; OB, osteoblasts; OC, osteocytes, BLC, bone lining cells, OD, odontoblasts.

programa ImageJ kao 95% (100% je crno), dok je najniži otkriven u ovom pokusu bio 23% (0% je bijelo). Druge dvije srednje razine izraženosti bile su slijedeće: signal u odonoblastima (OD) dobiven pomoću OC (80%) i signal u osteoblastima (OB) pomoću Col1 (45%). Slični rezovi hibridizirani istim probama, Col1 i OC, obilježeni radioaktivnošću pokazali su podjednaku izraženost Col1 i OC, prikaza-

(45%). Similar sections hybridized with the same probes, Col1 and OC, labeled with radioactivity showed the same expression pattern of Col1 and OC represented with distribution of silver grains as hybridization signal (Fig. 1). Using this radioactivity method, the localization of signal at the cell level was not as clear as with non-radioactive localization.



Slika 6. Kvantifikacija uz primjenu ImageJ različitih razina intenziteta hibridizacijskog signala pomoću neradioaktivne *in situ* hibridizacije. Relativna izraženost Collagen1a1 (A) i Osteocalcin (B) mRNA sa slike 5. kvantificirana je u odontoblastima i osteoblastima. Crna pruga na grafikonu predstavlja gotovo najviši mogući (95%) hibridizacijski signal dobiven pomoću Collagen1a1 mRNA u odontoblastima, označen tamno plavom strelicom kao na slici 5. Osteokalcinska mRNA izražena u odontoblastima, označena tamno sivom prugom u grafikonu i crvenom strelicom na slici 5. predstavlja još uvijek vrlo visok hibridizacijski signal (80% najvišeg signala), iako niži od prethodnoga u odontoblastima obilježenog pomoću Collagen1a1. Slijedeća svijetlo siva pruga u grafikonu koja pokazuje 45% najvišeg hibridizacijskog signala predstavlja izraženost uz Collagen1a1 u osteoblasima, označeno svijetlo plavom strelicom u slici 5. Još uvijek dobro vidljiv (23% najvišeg signala) hibridizacijski signal prikazan bijelom prugom na grafikonu predstavlja osteoblaste hibridizirane osteokalcinskom probom i označene svijetlo crvenom strelicom na slici 5. Zapaža se dobra korelacija hibridizacijskog signala, plave boje u slici 5. s mogućim rasponom kvantifikacije pomoću ImageJ u slici 6.

Figure 6. Quantification using ImageJ of different levels of hybridization signal intensities was obtained with non-radioactive in situ hybridization. The relative expression of Collagen 1a1 (A) and Osteocalcin (B) mRNA from Figure 5 was quantified in odontoblasts and osteoblasts. The black bar in the graph represents almost the highest possible (95%) hybridization signal which was obtained with Collagen 1a1 mRNA in odontoblasts and marked with the dark blue arrow as in Figure 5. The osteocalcin mRNA expressed in odontoblasts labeled with a dark gray bar in the graph and a red arrow in Figure 5 represents a still very high hybridization signal (80% of maximal signal), although lower than previously in odontoblasts labeled with Collagen 1a1. The next light gray bar in the graph showing 45% of maximum hybridization signal is represented with Collagen 1a1 expression in osteoblasts and labeled with a light blue arrow in Figure 5. The still highly visible (23% of maximum signal) hybridization signal is shown with a white bar graph representing osteoblasts hybridized with a osteocalcin probe and marked with light a red arrow in Figure 5. Note the good correlation of relative hybridization signal, blue color in Figure 5 with the possible range of ImageJ quantification in Figure 6.

no raspodjelom srebrnih zrnaca kao hibridizacijski signal (Slika 1.). Primjenom ove radioaktivne metode lokalizacija signala na staničnoj razini nije bila tako jasna kao kod neradioaktivne lokalizacije.

Osjetljivost metoda neradioaktivne *in situ* hibridizacije

Za ispitivanje osjetljivosti naših metoda neradioaktivne *in situ* hibridizacije u usporedbi s radioaktivnim *in situ* postupkom uz uporabu tkiva zuba i alveolarne kosti proveli smo hibridizaciju trima različitim neradioaktivnim metodama i radioaktivnom metodom uz primjenu triju različitih probi: BMP2, Col1 i OPN. Probu BMP2 rabili smo kao reprezentativnu za lokaliziranje jedne od mRNA prisutne u najmanjoj količini u kosti. Najbolji hibridizacijski signal neradioaktvnim *in situ* postupkom dobili smo pomoću 2. metode uz primjenu probe BMP2 obilježene digoksigeninom uz pojačavanje signala tiramidom i otkrivanje alkalnom fosfatazom (Slika 7B.). Intenzitet ovoga signala BMP2 usporediv je s 4. metodom, radioaktivnom *in situ* hibridizacijom (Slika 7D. i 7E.). Lokalizacija transkripata BMP2 mRNA uz pojačavanje signala tiramidom i otkrivanje fluorescencije cijaninom 5 (3. metoda) pokazala je minimalan hibridizacijski BMP2 signal (Slika 7C.). Neradioaktivni *in situ* postupak uz otkrivanje alkalnom fosfatazom bez pojačavanja (1. metoda) nije pokazao nikakav hibridizacijski signal za BMP2 (Slika 7A.). Ova 1. metoda nije bila dovoljno osjetljiva da bi obilježila rijetke transkripte BMP2 mRNA.

Probu Col1 rabili smo kao pozitivnu kontrolu, jer ona predstavlja jednu od mRNA prisutne u najvećoj količini u kosti. Hibridizacijski signal Col1 lokaliziran je svim ispitivanim *in situ* metodama u visokom intenzitetu (Slike 7F. do 7O.).

Probu OP rabili smo kao biljeg za osteocite, koštane stanice uklopljene u matriks. Ovaj gen je znatno manje obilan u kostima nego Col1, ali puno više od BMP2. Hibridizacijski signal za OP u osteocitima također smo našli svim ispitivanim *in situ* metodama (Slike 7F. do 7O.).

Rasprava

Razvili smo visoko osjetljivu metodu *in situ* hibridizacije na razini s radioaktibnim protokolima, s identifikacijskim fazama koje su presudne za lokaliziranje malog broja kopija mRNA u demineraliziranom tkivu uklopljenom u parafin, uz gotovo nepostojeću pozadinu i s mogućnošću kvantificiranja. Dosad je kvantificiranje za neradioaktivnu *in situ* hibridizaciju uz pomoć analizatora prikaza opisano samo na japanskom (24), iako je za radioaktivni *in situ* postupak objavljeno prije 22 godine (25).

Kako bismo ispitali snagu ove tehnike odabrali smo probe koje obilježavaju koštane stanice u zubima i mekom tkivu. Takve probe uključuju Col1, za koju se zna da je prisutna u

Sensitivity of non-radioactive *in situ* hybridization methods

To test sensitivity of our non-radioactive *in situ* hybridization methods with radioactive *in situ* using tooth and alveolar bone tissue, we performed hybridization with three different non-radioactive methods and the radioactive method using three different probes: BMP2, Col1 and OPN. The BMP 2 probe was used as a representative for localizing one of the lowest abundant mRNAs in the bone. The best hybridization signal, with non-radioactive *in situ*, was obtained with method 2 using BMP2 digoxigenin labeled probe with Tyramide Signal Amplification and alkaline phosphatase detection (Fig. 7B). The intensity of this BMP2 signal was comparable with method 4-radioactive *in situ* hybridization (Fig. 7D and 7E). Localization of BMP2 mRNA transcripts with Tyramide Signal Amplification and Cyanine 5 fluorescence detection, method 3, showed minimal BMP2 hybridization signal (Fig. 7C). Non-radioactive *in situ* using alkaline phosphatase detection without amplification, method 1, did not show any hybridization signal for BMP2 (Fig. 7A). This method 1 was not sensitive enough to label rare BMP2 mRNA transcripts.

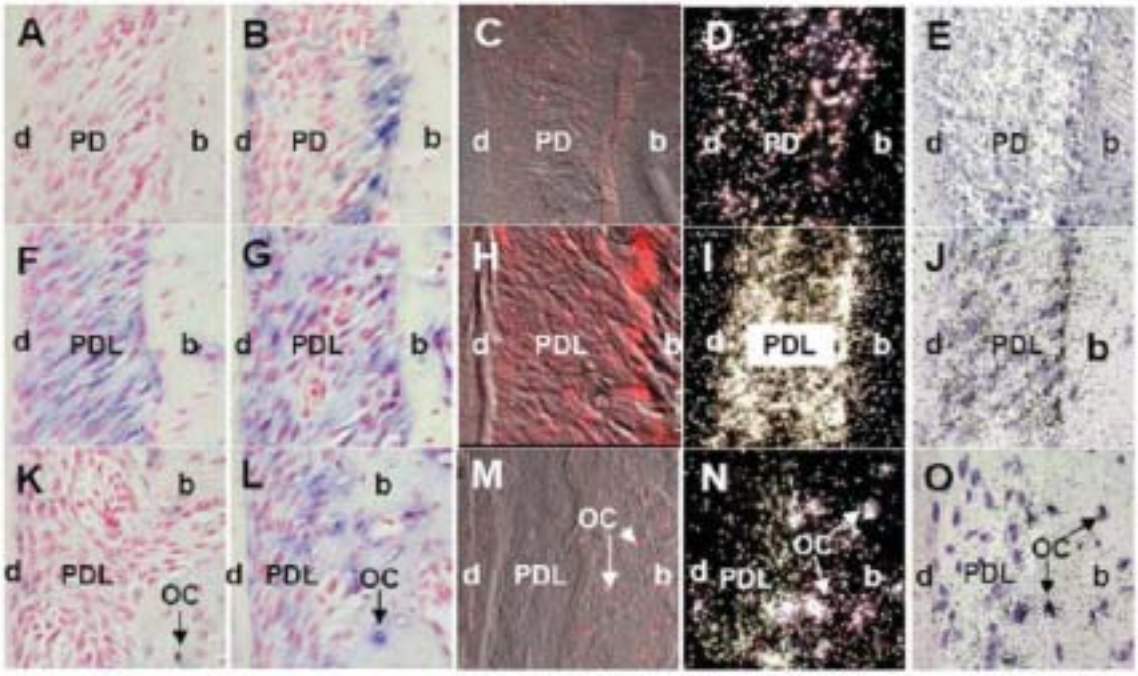
The Col1 probe was used as a positive control since it represents one of the most abundant mRNAs in bone. The hybridization signal of Col1 was localized with all tested *in situ* methods with great intensity (Fig. 7F to 7O).

OP probe was used as a marker for osteocytes, bone cells embedded in the matrix. This gene is much less abundant in bone than Col1, but much more than BMP2. The hybridization signal for OP in osteocytes was also found with all the *in situ* methods tested (Fig 7F to 7O).

Discussion

We have developed a high sensitive *in situ* hybridization method at a level with radioactivity, with identifying steps which are critical for localization of low copy number mRNAs in de-mineralized paraffin embedded tissue with almost nonexistent background and possibility of quantification. Up to date, quantification for non-radioactive *in situ* hybridization using image analyzer has been published, yet only by Japanese authors (24), while the one for radioactive *in situ* was published 22 years ago (25).

To test the power of this technique, we chose probes that label bone cells in teeth and soft tissue. Such probes include Col1 known to be abundant, whereas OC and OP are found at a lower copy number, but BMP2 are found in a very low copy number compared to Col1 in the same tissue. To achieve high sensitivity required for low-abundance mRNAs, we optimized crucial steps of hybridization and detection to achieve the best possible signal-to-noise ratio, comparable to radioactive *in situ* hybridization. All of these experiments were conducted on similar alveolar



Slika 7. Izraženost mRNA koštanog morfogenetskog proteina 2 (BMP2) (A-E), Collagen1a1 (Col1) (F-J) i osteopontina (OP) (K-O) u reprezentativnim rezovima mišje maksile izvedena pomoću četiri različite metode *in situ* hibridizacije: 1. neradioaktivnim *in situ* postupkom pomoću digoksigeninom obilježenih probi uz otkrivanje alkalnom fosfatazom (A, F, K); 2. neradioaktivnim *in situ* postupkom pomoću digoksigeninom obilježenih probi uz pojačavanje signala tiramidom i otkrivanje alkalnom fosfatazom (B, G, L); 3. neradioaktivnim *in situ* postupkom pomoću digoksigeninom obilježenih probi uz pojačavanje signala tiramidom i cijanin 5 flurescenciju (C, H, M); i 4. radioaktivnim *in situ* postupkom pomoću ^{32}P obilježenih probi i autoradiografskim otkrivanjem (D, I, N, snimci u tamnom polju; E, J, O, snimci u svijetlom polju).
Kod neradioaktivne *in situ* hibridizacije uz otkrivanje alkalnom fosfatazom (1. i 2. metoda) pozitivni hibridizacijski signal je predstavljen plavom bojom, a crvena boja je kontrabojenje nuklearnog crvenog (A, B, F, G, K, L). Kod neradioaktivne *in situ* hibridizacije pomoću fluorescencije cijaninom 5 za otkrivanje (4. metoda) pozitivni signal je predstavljen svijetlo crvenom bojom (C, H, M). Bijela srebrnasta zrnca predstavljaju pozitivan hibridizacijski signal na autoradiografima radioaktivne *in situ* hibridizacije (3. metoda) primjenom prikaza u tamnom polju (D, I, N), a crna srebrnasta zrnca primjenom prikaza u svijetlom polju (E, J, O).
Najbolji hibridizacijski signal za probu BMP2 dobiven je (2. metoda) neradioaktivnim *in situ* postupkom pomoću digoksigeninom obilježenih probi uz pojačavanje signala tiramidom i otkrivanje alkalnom fosfatazom (B) i (4. metoda) radioaktivnom *in situ* hibridizacijom (D, E). Zapaža se prednost neradioaktivne lokalizacije BMP2 mRNA na razini stanica (B) u usporedbi s radioaktivnom (D, E). Lokalizacija transkripata BMP2 mRNA pomoću pojačavanja signala tiramidom i cijanin 5 fluorescencije (3. metoda) pokazala je minimalan hibridizacijski signal za BMP2 (C). Neradioaktivni *in situ* postupak uz otkrivanje alkalnom fosfatazom bez pojačavanja (1. metoda) nije pokazao nikakav hibridizacijski signal za BMP2 (A).
Hibridizacijski signal za probu Col1 lokaliziran je svim četirima *in situ* metodama (F-O).
Hibridizacijski signal za OP u osteocitima također je nađen svim četirima *in situ* metodama (F-O).
Opis obilježavanja: d, dentin; PD, periodont; b, kost; OC, osteociti.

Figure 7. The representative sections of mouse maxilla showing BMP2-Bone Morphogenetic Protein 2 (A-E), Col1-Collagen 1a1 (F-J) and OP-osteopontin (K-O) mRNA expression performed with four different in situ hybridization methods: 1) non-radioactive in situ using digoxigenin labeled probes with alkaline phosphatase detection (A, F, K); 2) non-radioactive in situ using digoxigenin labeled probes with Tyramide Signal Amplification and alkaline phosphatase detection (B, G, L); 3) non-radioactive in situ using digoxigenin labeled probes with Tyramide Signal Amplification and Cyanine 5 fluorescence (C, H and M); and 4) radioactive in situ using ^{32}P labeled probes and autoradiography detection (D, I and N, darkfield images; E, J and O, bright field images).
In non-radioactive in situ hybridization with alkaline phosphatase detection (methods 1 and 2), positive hybridization signal is represented with blue stain and red color is counterstain from nuclear red (A, B, F, G, K and L). In non-radioactive in situ hybridization using fluorescence of Cyanine 5 as detection (method 4), positive signal is represented with bright red color (C, H and M). The white silver grains represent positive hybridization signal in autoradiographs of radioactive in situ hybridization (method 3) using dark field images (D, I and N), and black silver grains using bright field images (E, J and O).
The best hybridization signal for BMP 2 probe was obtained with (method 2) non-radioactive in situ using digoxigenin labeled probes with Tyramide Signal Amplification and alkaline phosphatase detection (B) and (method 4) radioactive in situ hybridization (D, E). Note advantage of non-radioactive localization of BMP2 mRNA at the level of cells (B), compared to the radioactive one (D, E). Localization of BMP2 mRNA transcripts with Tyramide Signal Amplification and Cyanine 5 fluorescence (method 3) showed minimal BMP2 hybridization signal (C). Non-radioactive in situ using alkaline phosphatase detection without amplification (method 1) did not show any hybridization signal for BMP2 (A).
Col1 probe hybridization signal was localized with all four in situ methods (F-O).
The hybridization signal for OP in osteocytes was also found with all four in situ methods (F-O).
Labeling description: d, dentin; PD, periodontium; b, bone; OC, osteocytes.

velikoj količini, dok se OC i OP nalaze uz manji broj kopija, ali se BMP2 nalazi u vrlo malom broju kopija u usporedbi s Col1 u istom tkivu. Kako bismo postigli visoku osjetljivost potrebnu za mRNA prisutnu u maloj količini, optimirali smo presudne faze hibridizacije i otkrivanja da bismo postigli najbolji mogući omjer signala i buke, usporediv s radioaktivnom *in situ* hibridizacijom. Svi ovi pokusi izvedeni su na sličnim rezovima alveolarne kosti s koštanim, zubnim i periodontnim PDL kao mekim tkivom. To nam je omogućilo laku i jasnu usporedbu hibridizacijskog signala u istim stanicama te između radioaktivne i neradioaktivne hibridizacijske tehnike.

Utvrdili smo kako su slijedeće faze neradioaktivne *in situ* hibridizacije presudne:

1. kvaliteta fiksiranog tkiva;
2. obilježavanje probe i određivanje koncentracije;
3. prodiranje probe u tkivo;
4. odgovarajuće razrjeđenje anti-digoksigeninskih antitijela;
5. dostatna duljina razvijanja boje kod enzimatske reakcije.

Dobra kvaliteta tkiva za *in situ* hibridizaciju čuva cjelovitost ciljne mRNA u tkivu uz primjenu odgovarajuće fiksacije bez RNAza, što daje najbolji hibridizacijski signal. Premala fiksacija, međutim, može dovesti do gubitka tkiva i mRNA, dok prekomjerna fiksacija može uzrokovati pretjerano križno povezivanje, što može citoplazmu učiniti relativno nepropusnom za sve probe.

Svi naši protokoli obuhvaćaju postupke sasvim bez RNAza: predhibribridizacijske i hibridizacijske reagense uključujući posude i sve suđe koje će doći u doticaj s tkivom prije ili tijekom hibridizacije. Postupci su već prije opisani (17). Ove mjere opreza u vezi s RNAza su bitni, jer onečišćenje RNAzom može potpuno uništiti ciljne mRNA.

Priprema probi je veoma važna. Prije transkripcije digoksigeninom plazmide koji sadrže umetke cDNA probe treba linearizirati odgovarajućom restrikcijskom endonukleazom kako bi se stvorila samo sekvenca cDNA probe. Važno je da se restrikcijska digestija provede u potpunosti. Mala količina nedigestirane plazmidne DNA može rezultirati ne samo transkriptima probe, nego i vrlo dugim transkriptima cijelog plazmida koji mogu sadržavati znatan odsječak digoksigeninom obilježenog rNTP. Takva vrst obilježene probe neće biti specifična i neće hibridizirati specifične ciljne mRNA. Hibridizacijsko *in situ* obilježavanje neće postojati ili neće biti specifično. Slični će se rezultati dobiti ako kalup koji se rabi za *in vitro* transkripciju ne nosi izbočene 5′ krajeve ili ako nemaju slijepe završetke, kako je opisano u literaturi (26). Zbog ovih smo razloga izbjegavali primjenu enzima koji stvaraju izbočene 3′ krajeve, kao što su: Aat II, Bgl I, Cfo I, Hha I, Pvu I, Sfi I, Apa I, Bsp 1286 I, Hae II, Kpn I, Sac I, Sph I, Ban II, Bstx I, HgiA I, Pst I i Sac II. Ako nismo mogli naći odgovarajući enzim,

bone sections with bone, teeth and (periodontium) PDL as soft tissue. This allowed us easy and clear comparison of hybridization signal in the same cells and between radioactive and non-radioactive hybridization techniques.

Non-radioactive *in situ* hybridization steps identified to be crucial are:

1. quality of the fixed tissue;
2. probe labeling and determination of concentration;
3. penetration of the probe into the tissue;
4. appropriate dilution of Anti-digoxigenin antibodies; and
5. adequate length of developing color with enzymatic reaction.

Good quality of tissue for *in situ* hybridization preserves the integrity of target mRNA in the tissue using RNase free appropriate fixation which gives the best hybridization signal. Little fixation, however, can lead to loss of tissue and mRNA, and too much can cause extensive cross linking that may render the cytoplasm relatively impermeable to all probes.

All our protocols involve complete RNase free: pre-hybridization and hybridization reagents including dishes and any glassware which will be in contact with tissue before or during hybridization. Procedures have been described elsewhere (17). These RNase precautions are crucial since RNase contamination can completely destroy mRNA targets.

The preparation of probe is very important. Before transcription with digoxigenin, plasmids containing cDNA probe inserts should be linearized with appropriate restriction endonuclease enzyme to produce the cDNA probe sequence only. It is important that the restriction digestion be performed to completion. A small amount of undigested plasmid DNA can give rise not only to probe transcripts but also to very long whole plasmid transcripts which may incorporate a substantial fraction of digoxigenin labeled rNTP. This kind of labeled probe will not be specific and will not hybridize specific target mRNAs. Hybridization *in situ* labeling will be nonexistent or nonspecific. Similar results will exist if the template used for *in vitro* transcription does not carry protruding 5′ termini or are not blunt ended as reported by Schenborn and Mierendorf (26). For these reasons, we avoided using enzymes creating 3′ protruding termini such as: Aat II, Bgl I, Cfo I, Hha I, Pvu I, Sfi I, Apa I, Bsp 1286 I, Hae II, Kpn I, Sac I, Sph I, Ban II, Bstx I, HgiA I, Pst I and Sac II. If we could not find an appropriate enzyme and we digested with enzymes creating recessed 3′ protruding termini, these ends need to be filled-in by the polymerase activity of Klenow fragment of *E. coli* DNA polymerase I, in the presence of appropriate deoxynucleotides (dNTPs) (17). We think that other reasons that the transcription reaction fails or is poor are:

digestiju smo izvodili s enzimima koji stvaraju uvučene 3' izbočene krajeve, takve završetke treba ispuniti polimeraznom aktivnošću Klenowljeva odsječka DNA polimeraze I *E. coli* u prisutnosti odgovarajućih deoksinukleotida (dNTP) (17). Smatramo kako su ostali razlozi zbog kojih transkripcijska reakcija izostane ili je slaba slijedeći:

1. odrezana plazmidna DNA koja se rabi kao kalup za transkripciju nije dovoljno čista, stoga treba provesti ponovnu ekstrakciju fenolom/kloroformom i spektrofotometrom provjeriti koncentraciju i čistoću;
2. previše je DNA u transkripcijskoj reakciji, pa je potrebna manja količina DNA;
3. upotrebljena je kriva polimeraza, stoga treba primijeniti ispravnu polimerazu.

Drugi problem može nastati ako proba nije potpuno otopljena i resuspendirana. Prema proizvođačima, nešto je teže raditi s RNA obilježenom digoksigeninom nego s redovnom RNA, vjerojatno zbog hidrofobičnosti haptena što ga čini manje topljivim. Zbog toga ekstrakcija RNA obilježene DIG fenolom/kloroformom dovodi do većih gubitaka i treba ju izbjegavati; međutim, precipitate RNA obilježene DIG može ponekad biti teško ponovno otapati i to može zahtijevati zagrijavanje do 65 °C kroz 20 minuta uz vorteksiranje, miješanje i/ili dodavanje SDS do 0,1% kako bi se pripomoglo topljivosti.

Kod *in situ* hibridizacije sposobnost probi da prožmu rezove ima velik učinak na jačinu hibridizacijskog signala. Za postizanje najučinkovitijeg signala primjenjuju se tri različite tehnike: s Tritonom, proteazom i proteinazom K (18,27-29). Prema našem iskustvu, Triton dolazi u obzir za smrznute rezove, no za parafinske rezove je proteinaza K pravi izbor. Mi smo upotrijebili 5 μg/mL za obje metode (19). Postoji stanovita dogma za *in situ* hibridizaciju na rezovima, prema kojoj za dobar prodor duljina obilježene RNA probe treba biti kratka; stoga je optimalna duljina probe 50–150 baznih parova (18). Međutim, obilježena RNA proba može se obraditi blagom lužinom kako bi se hidrolizirala na optimalnu duljinu (valja napomenuti kako se ovom obradom ne uništava digoksigeninski hapten). Međutim, mi smo ustanovili kako smanjenje veličine probe možda i nije tako važno u ovom hibridizacijskom postupku sve dok je veličina probe manja od 1 kb (10).

Iako se prema postojećim protokolima za neradioaktivnu *in situ* hibridizaciju s digoksigeninom rabe različita razrjeđenja anti-digoksigeninskim antitijela, o toj se količini ili razrjeđenju nije raspravljalo. Mi smo ustanovili kako je nemoguće otkriti mali broj kopija ciljne mRNA uz razrjeđenje 1:5000 anti-digoksigeninskih antitijela (odsječci anti-DIG Fab), kako to preporučuju neki *in situ* protokoli. Otkrivanje hibridizacijskog signala uvelike ovisi o kopijama mRNA razrjeđenju odjsečaka anti-DIG Fab. Proizvođač za *in situ* hibridizaicju savjetuje 1:20 do 1:100, ali većina protokola rabi veća razrjeđenja. Možda razlozi visoke

1. the cut plasmid DNA used as template for transcription is not pure enough, therefore re-extraction with phenol/chloroform and checking with a spectrophotometer for the concentration and purity needs to be done;
2. there is too much DNA in the transcription reaction, therefore less amount of DNA is needed; and
3. a wrong polymerase was used, therefore the correct one needs to be used.

Another problem can arise if the probe is not completely dissolved and resuspended. According to manufacturers, digoxigenin labeled RNA is slightly more difficult to work with than regular RNA and is probably due to hydrophobicity of the hapten making it less soluble. Because of this, phenol/chloroform extraction of DIG labeled RNA results in major losses and should be avoided; however, precipitates of DIG labeled RNA can sometimes be difficult to redissolve and may require heating to 65 ˚C for 20 minutes by vortexing, mixing, and/or the addition of SDS to 0.1%, to aid solubility.

In the *in situ* hybridization, the ability of the probes to permeate sections has a major effect on the strength of hybridization signal. To achieve the most efficient signal, three different techniques are used: with Triton, protease and Proteinase K (18, 27-29). From our experience, Triton is considered for frozen sections but for paraffin sections Proteinase K is the treatment of choice. We used 5 μg/mL for both methods (19). There is a dogma for *in situ* hybridization on sections that good penetration of the labeled RNA probe length should be short; thus, the optimum probe length is 50-150 base pairs (18). However, the labeled RNA probe can be treated with mild alkali to hydrolyze for optimum length (note that digoxigenin hapten is not destroyed by this treatment). However, we found that probe size reduction may not be so important in this hybridization procedure as long as the probe size is less than 1 kb (10).

In the existing protocols for non-radioactive *in situ* with digoxigenin, different dilutions of anti digoxigenin antibodies are used and this amount or dilution was not discussed. We found out that trying to detect few copies of target mRNA is impossible with a 1:5000 dilution of anti-digoxigenin antibodies (anti-DIG Fab fragments), as recommended in some *in situ* protocols. Detection of hybridization signal is greatly dependent upon the mRNA copies and dilution of anti-DIG Fab fragments. The manufacturer's suggestion for *in situ* hybridization is 1:20 to 1:100 but most of the protocols use higher dilutions. Maybe the reasons for high background became a problem in some tissues with low antibody dilution and developing time longer than 1 hour. We also think that this is very important, during developing color more than 1 hour, to add 2 mM Levamisole, an endogenous alkaline phospha-

pozadine postaju problem u nekim tkivima uz nisko razrjeđenje antitijela i vrijeme razvijanja duže od 1 sata. Mi također mislimo kako je kod razvijanja boje dužeg od 1 sata veoma važno dodati 2 mM levamisola, endogenog inhibitora alkalne fosfataze, kako je to prije preporučeno (27) i 10 mM N-etil maleimida za inhibiciju reakcije „ništa dehidrogenaze" (22).

Duljina vremena razvijanja obojene reakcije kod digoksigeninske *in situ* hibridizacije veoma je važna za kvantifikaciju. Za kvantifikaciju treba treba pažljivo motriti razvijanje boje, jer prekomjerno razvijanje boje može dostići najviši mogući intenzitet i podcijeniti najvišu izraženost u usporedbi s drugim nižim izražajnostima mRNA koje s vremenom još uvijek razvijaju boju. Ranije su se *in situ* kvantifikacije radile samo radioaktivnim i fluorescentnim metodama. Mi smo ovdje pokazali kako našom metodom neradioaktivne *in situ* hibridizacije možemo kvantificirati različite razine plavog obojenja koje predstavljaju različite razine izražajnosti mRNA te ovu izražajnost uspoređivati u rasponu od 0–100% ili u formatu od 8 bitova od 0 do 256. Ovo je dosad prvi opisani protokol neradioaktivne *in situ* kvantifikacije.

Naši rezultati pokazuju sličan model *in situ* hibridizacije kao kod ranije opisane radioaktivne i neradioaktivne *in situ* hibridizacije (30). Naša 2. metoda neradioaktivne *in situ* hibridizacije pokazuje hibridizacijski signal kod BMP2, mRNA prisutne u vrlo maloj količini, jednako učinkovit kao signal kod radioaktivne metode. Mi smatramo da su probe obilježene digoksigeninom (ili druge haptenom obilježene probe) bolje u usporedbi s radio-obilježenim probama za svaki pokus, i to zbog stabilnosti probe i mogućnosti njezina čuvanja mjesecima (31). Ostale prednosti neradioaktivnih probi su brže vrijeme do postizanja rezultata i manji broj dana za postupak s digoksigeninom u usporedbi s 3 tjedna za radioaktivni postupak (30). Daljnja prednost je bolja prostorna rezolucija otkrivanja enzima pomoću neradioaktivnih postupaka negoli s radioaktivnošću, što dopušta precizniju lokalizaciju ciljne sekvence (vidi Slike 3., 4., 5. i 7). Nadalje, sve ispitane tehnike neradioaktivne hibridizacije pokazuju jedinstvenu izraženost na razini jedne stanice bez pozadine. Ovdje opisani neradioaktivni postupci ispitani su s velikim uspjehom uz više drugih probi (Slika 7.). Mi smo lokalizirali BMP2 mRNA, poznatu kao jednu od vrlo rijetkih u kosti, pomoću 2. metode (Slika 7B.), uz intenzitet radioaktivno *in situ* postupka (Slike 7D. i 7E.). Uz primjenu iste probe lokalizirali smo BMP2 u istom tkivu 3. metodom uz nizak intenzitet (Slika 7C.), dok 1. metodom nismo lokalizirali BMP2 (Slika 7A.). Osjetljivost 2. metode jednaka radioaktivnoj metodi (4. metoda) postiže se dvama tehničkim koracima: jedan korak je dodavanje polivinil alkohola (PVA) koji se rabi za pojačanje razvijanja boje tijekom reakcije otkrivanja alkalne fosfataze, gdje BICP (supstrat AP) nakon defosforilacije daje tamno-plavu indigo boju kao oksidacijski produkt koji se dalje pojačava oksidacijom NTB, druge komponente AP supstrata. PVA sprječava difuziju tamno-plavog indigo-dye kao AP reakcijskog produkta i to je ono što

tase inhibitor as recommended before (27) and 10 mM N-ethyl maleimide (to inhibit "nothing dehydrogenase" reaction) (22).

The amount of time for developing color reaction in digoxigenin *in situ* hybridization is very important for quantification. In order to do quantification, development of colors should be monitored closely since overdeveloping color can reach highest possible intensity and under-estimate the highest expressions in comparison with other lower mRNA expressions still developing color with time. Previously, *in situ* quantifications were done only with radioactive and fluorescent methods. Here we have shown that with our non-radioactive *in situ* hybridization method, we can quantify different levels of blue staining that represent different levels of mRNA expression, and compare this expression in the range of 0-100% or in an 8 bit format of 0 to 256. Up to date, this is the first non-radioactive *in situ* quantification protocol described.

Our results demonstrate a similar *in situ* hybridization pattern with radioactive and non-radioactive *in situ* hybridization as reported previously (30). Our non-radioactive *in situ* hybridization method 2 shows hybridization signal, of very low abundant BMP2 mRNA, as signal efficient as the radioactive method. We think that digoxigenin labeled probes (or other hapten labeled probes) are superior compared to radio-labeled for each experiment, due to the probe stability and possibility of its storage for many months (31). Other advantages of non-radioactive probes are the faster turnaround time of achieving results, and few days for digoxigenin procedure compared to 3 weeks for the radioactive procedure (30). Another advantage is spatial resolution of the enzyme detection using non-radioactive procedures as being much better than can be obtained with radioactivity, allowing for more precise target sequence localization (see Figs. 3, 4, 5 and 7). Furthermore, all tested non-radioactive hybridization techniques show unique expression at the level of single cell without background. The non-radioactive procedures described herein have been tested by several other probes with great success (Fig. 7). We localized BMP2 mRNAs, known as one of the very rare in bone, using method 2 (Fig. 7B), with intensity of radioactive *in situ* (Fig. 7D and 7E). Using the same probe, we localized BMP2 in the same tissue with very low intensity, with method 3 (Fig. 7C) but with method 1, BMP2 was not localized (Fig. 7A). Sensitivity of method 2, equivalent to radioactive method (method 4) is achieved with two technical steps. One step is the addition of polyvinyl alcohol (PVA) used as color development enhancer during alkaline phosphatase detection reaction, where BICP (AP substrate) after de-phosphorylation gives dark-blue indigo-dye as an oxidation product further intensified with oxidation of NTB, another component of AP substrate. PVA prevents diffusing of dark-blue indigo-dye as AP reaction product and this is what

izvod koji se nadalje intenzivira oksidacijom NTB, druge sastavnice supstrata AP. PVA priječi difuziju tamno-plave indigo boje kao proizvoda reakcije AP i to je ono što pojačava obojeni signal. Drugi korak je primjena TSA (pojačavanje signala tiramidom), čime se značajno pojačavaju kromogeni i fluorescentni signali. Za *in situ* otkrivanje signala tehnologija TSA rabi hrenovu peroksidazu (HRP) pripojenu digoksigeninskim antitijelima vezanima za digoksigeninom obilježene probe za lokaliziranje mRNA, kako bi katalizirala odlaganje dinitrofenilom (DNP) obilježen reagens za pojačavanje neposredno uz imobilizirani enzim peroksidazu iz hrena. Ova DNP obilježja mogu se tada indirektno otkriti pomoću anti-DNP-alkalna fosfataza antitijela i konačno slijedi enzimsko otkrivanje boje. Fluorescentna 3. metoda također upotrebljava tehnologiju TSA. Ovom metodom dobili smo *in situ* hibridizacijski signal pomoću BMP2 probe, relativno slabo zastupljen u kosti. Intenzitet signala pomoću 3. metode bio je niži nego uz pomoć 2. metode. Razlog: 3. metoda rabi fluorescentno otkrivanje, a ne alkalnu fosfatazu. Fluorescentna 3. metoda također rabi reagens TSA, ali izravno obilježen fluoroforom, što pojednostavljuje otkrivanje za neposredno prikazivanje fluorescentnim mikroskopom. Otkrivanje alkalnom fosfatazom za 2. metodu je osjetljivije nego fluorescentno otkrivanje, jer je nakon reagensa TSA konačno otkrivanje neizravno, uz primjenu dodatnih anti-DNP-alkalna fosfataza antitijela i razvijanja PVA uz AP pojačavanje boje, što traje od 30 minuta do preko noći, a oboje dodatno povećava osjetljivost. Pomoću 1. metode nismo lokalizirali BMP2; ova metoda rabi osjetljivo otkrivanje boje enzimom AP, ali ne rabi TSA umnažanje, što značajno pojačava *in situ* signal. Naša je procjena da je neradioaktivna 2. metoda najmanje 10 puta osjetljivija od neradioaktivne 1. metode i 3-5 puta osjetljivija od 3. metode. Prednost neradioaktivne lokalizacije BMP2 mRNA pomoću 2. metode je signal na razini stanica (slika 7B.), u usporedbi s radioaktivnom (slike 7D. i 7E.). Razlog za nepreciznu lokalizaciju radioaktivnog signala na razini stanice je širenje energije ^{32}P na okolna područja, što utječe na izloženost fotografske emulzije koja se rabi za otkrivanje hibridizacijskog signala. Rezultat je mnoštvo raspršenih srebrnih zrnaca na izvoru signala radioaktivne hibridizacije i oko njega, što omogućava otkrivanje na razini jedne stanice.

U našem konačnom ispitivanju tehnike *in situ* hibridizacije rabili smo osteopontinsku (OP) probu kao biljeg relativno slabo prisutne mRNA za osteocite – koštane stanice uklopljene u matriks. Pomoću ove probe lokalizirali smo signale u osteocitima svim neradioaktivnim metodama (Slika 7K., 7L. i 7M.). Intenzitet dobivenog signala bio je usporediv s onim dobivenim radioaktivnom metodom (Slika 7N. i 7O.), dajući tako konačan dokaz korisnosti naših metoda neradioaktivne *in situ* hibridizacije u različitim stanicama i tkivima.

enhances the color signal. The second step is the use of TSA (Tyramide Signal Amplification) that significantly enhances chromogenic and fluorescence signals. For *in situ* signal detection, TSA technology uses horse radish peroxidase (HRP), attached to anti digoxigenin antibodies bound to digoxigenin labeled probes localizing mRNA, to catalyze the deposition of dinitrophenyl (DNP) labeled amplification reagent adjacent to the immobilized HRP enzyme. These DNP labels can then be indirectly detected using anti-DNP-Alkaline Phosphatase antibodies and finally AP enzyme color detection. Fluorescent method 3 also uses TSA technology. With this method, we obtained an *in situ* hybridization signal using the BMP2 probe, relatively low abundant message in bone. Signal intensity using method 3 was lower than using method 2. Reason: method 3 uses fluorescent detection rather than AP. The fluorescent method 3 also uses TSA reagent but directly labeled with fluorophore simplifying the detection for immediate visualization *via* fluorescence microscope. AP enzyme detection for method 2 is more sensitive than fluorescence since after TSA reagent final detection is indirect, using additional anti-DNP-Alkaline Phosphatase antibodies and PVA development AP color enhancement lasting from 30 minutes to overnight, both additionally increasing sensitivity. With method 1 we did not localize BMP2; this method uses sensitive color AP enzyme detection but does not use TSA amplification which enhances *in situ* signal significantly. Our estimation is that non-radioactive method 2 is at least 10 times more sensitive than non-radioactive method 1 and 3-5 times more sensitive than method 3. The advantage of non-radioactive localization of BMP2 mRNA with method 2 is signal at the level of the cells (Fig. 7B), compared to radioactive one (Fig. 7D and 7E). The reason for non-precise localization of radioactive signal at the cell level is spreading high ^{32}P energy to surrounding areas, effecting exposure of photograph emulsion used for hybridization signal detection. Result is a lot of scattered silver grains, at and around the source of radioactive hybridization signal, which enables detection at the single cell level.

Our final *in situ* hybridization technique testing used osteopontin (OP) probe as a relatively abundant mRNA marker for osteocytes, i.e. bone cells embedded in matrix. Using this probe, we localized signals in osteocytes with all non-radioactive methods (Fig. 7K, 7L and 7M). Obtained signal intensity was comparable to the radioactive method (Fig. 7N and 7O) and provided final proof of usefulness of our non-radioactive *in situ* hybridization methods in a variety of cells and tissues.

We feel that the use of these non-radioactive *in situ* hybridization analyses with our new quantification protocol offers greater potential for novel biological insights in many fields including research and clinical chemistry.

Smatramo kako primjena ovih neradioaktivnih *in situ* hibridizacijskih analiza uz naš novi protokol kvantifikacije nudi veće mogućnosti za stjecanje novih bioloških saznanja u mnogim područjima uključujući istraživanje i kliničku kemiju.

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Adresa za dopisivanje:

Jelica Gluhak-Heinrich
Department of Orthodontics
The University of Texas Health Science Center at San Antonio
7703 Floyd Curl Dr.
San Antonio
TX 78229-3900
USA
e-pošta: gluhak@uthscsa.edu
tel: +1 210 5673 509
faks: +1 210 5672 614

Corresponding author:

Jelica Gluhak-Heinrich
Department of Orthodontics
The University of Texas Health Science Center at San Antonio
7703 Floyd Curl Drive
San Antonio, TX 78229-3900
USA
e-mail: gluhak@uthscsa.edu
phone: +1 210 5673 509
fax: + 1 210 5672 614

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