

Formaldehyde cross-linking of proteins to osteoclast differentiation factor promoter for the identification of biofunctional proteins

Mojgan Haghighi, M.Eng, PhD.

ABSTRACT

الأهداف: إيجاد الحالة المثلى للربط البيني للعامل النووي (NFY) (A, B, C) وإيجاد عوامل الاستنساخ التي تتعلق بظهور (ODF).

الطريقة: أجريت هذه التجربة من أجل البحث في حالة ربط العامل النووي (NFY)، تم اكتشاف الربط البيني لعقار فورمالديهايد، وتم تطهير المركب باستعمال الترسيب المناعي. تم اكتشاف بعض البروتينات كعوامل داخلية. كما تم اكتشاف التفاعل بين (NFY) ومحرض (ODF) باستعمال تفاعل سلسلة الخماثر الناقلة المتعددة (PCR).

النتائج: تم تحديد حالة الربط البيني المثلى للعامل النووي (NFY) باستعمال 10 عينات حسب التالي: 0.7% إلى 1.3% من عقار فورمالديهايد و 3-15 دقيقة من وقت الحضانة. أظهرت النتائج تفاعل (NFY) (A, B, C) مع بعضها محرض (ODF) المرتبط وتداخل ذلك مع العوامل الأخرى مثل (VDR) في ظهور (ODF).

خاتمة: الربط البيني الأمثل مبني بشكل رئيسي على تركيز البروتين، (pH)، المواد المضافة، درجة الحرارة، عدد المجموعات المتفاعلة، طول ذراع الحيز وحجم الفاصل والتركيب.

Objective: To find the optimal cross-linking condition for nuclear factor (NF)Y (A, B, C), and find the transcription factors involved in osteoclast differentiation factor (ODF) expression.

Methods: This experiment was carried out from 2002 to 2003 in Handa Lab, Faculty of Bioscience and Biotechnology, Tokyo Institute of Technology, Tokyo, Japan. To search for the NFY-binding condition, optimal formaldehyde cross-linking was detected, and the complex was purified by immunoprecipitation. Some proteins were detected as interactors. Interaction between NFYs and the ODF promoter was detected by polymerase chain reaction.

Results: The optimal cross-linking condition for NFYs was determined using 10 samples with 0.7-1.3% formaldehyde, and 3-15 minutes incubation time. The results showed the interaction of NFY A, B, and C with each other for ODF promoter binding and involvement of other factors like vitamin D receptor in ODF expression.

Conclusion: Optimal cross-linking conditions vary, based on protein concentration, pH, additives, temperature, number of reactive groups, spacer arm length, and buffer volume and composition.

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From the Department of Biological Informatics, Faculty of Bioscience and Biotechnology, Tokyo Institute of Technology, Tokyo, Japan.

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Address correspondence and reprint request to: Dr. Mojgan Haghighi, Department of Biomedical Informatics, Faculty of Medicine, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku 113-8549, Tokyo, Japan. Tel. +81 (3) 58034696. E-mail: haghighi@bioinfo.tmd.ac.jp

Bone diseases are one of the common problems in old age, especially among post-menopausal women. For this reason, it is important to identify genetic factors involved in these diseases. There are 3 special types of cells that are found only in the bone: osteoblasts, osteoclasts, and osteocytes. Osteoclasts are large cells, which dissolve the bone and are related to white blood cells, they are formed from 2 or more cells that fuse to each other, and are found in the surface of the bone, the mineral next to the dissolving bone. Osteoblasts are the cells that form new bones called "osteoids," which are made of bone collagen. They control calcium and mineral deposition. The third group are cells inside the bone called osteocytes. Osteoblasts turn into osteocytes, while the new bone is being formed. Osteoblasts and osteoclasts are normally balanced in their activity, but some factors including the expression of osteoclast

differentiation factor (ODF) can increase osteoclast maturation and differentiation, as was shown in the experimental models of osteolytic metastases such as, primary bone tumors, humoral hypercalcemia of malignancy, osteoporosis, rheumatoid arthritis, and osteolysis. Osteoclast differentiation factor expression is stimulated by agents including hydroxide (OH⁻), vitamin D, parathyroid hormone, as well as, parathyroid hormone related peptide, interleukin (IL)-6, and IL-11. Osteoprotegerin (OPG) inhibits ODF/osteoclast differentiation factor receptor (ODFR) complex formation, and inhibits osteoclast activation. The recombinant receptor activator of nuclear factor- κ B (RANK)-L antagonist, RANK-Fc (the soluble form of ODFR) acts to sequester endogenously produced ODF. Various transcription factors trigger ODF expression,^{1,2} some of them known and some unknown, such as, vitamin D receptor (VDR) induces ODF expression by forming a heterodimer with retinoid x receptors, which then interacts with a vitamin D-responsive element (VDRE). The VDRE then either promotes, or inhibits the transcription of ODF messenger ribonucleic acid. The nuclear factor (NFY) complex is one of the known inducers of ODF expression. It has 3 subunits: NFY A (45 kiloDaltons [kDa]), NFY B (34 kDa) and NFY C (43 kDa). These complexes bind to the CCAATCT sequence of the ODF promoter, called the CCAATCT box. The NFYs have some interactions with general transcription factors (TF), such as: TATA binding protein (TBP), TFIIF, tumor angiogenic factors, and other transcription factors. To discover the interactors, we tried to cross-link proteins by using formaldehyde.

Properties of formaldehyde and its polymers. Formaldehyde is a gas whose small molecules (HCHO), of which the -CHO is the aldehyde group, dissolve rapidly in water, with which they chemically combine to form methylene hydrate. This is the form in which formaldehyde exists in aqueous solutions, and its chemical reactivity is similar to formaldehyde gas.

Reaction between proteins and formaldehyde. The aldehyde group can combine with nitrogen and some other atoms of proteins, or with 2 such atoms if they are very close together, forming a cross-link, called a methylene bridge. Studies indicate that the most common type of cross-linking formed by formaldehyde in collagen is between the nitrogen atom at the end of the side-chain of lysine, and the nitrogen atom of the peptide linkage, and that the number of such cross-links increases with time. The tanning of collagen to make leather is comparable to the hardening of a tissue by a fixative. The fixative action of formaldehyde is probably related to its reaction with proteins. When a specimen is dehydrated after only a few hours in formaldehyde, the largely unfixed cytoplasmic proteins are coarsely coagulated. Nuclear chromatin, which contains deoxyribonucleic acid, and strongly basic proteins, is also

coagulated by the solvent, forming a pattern of threads, lumps, and granules. The combination of formaldehyde with glutaraldehyde as a fixative for electron microscopy takes advantage of the rapid penetration of the small HCHO molecules, which initiate the structural stabilization of the tissue and chromatin. The aim of this study is to find transcription factors which are involved in ODF expression, and to find optimal cross-linking condition of one of these transcription factors by using formaldehyde.

Methods. This experiment was carried out from 2002-2003 in Handa Laboratory, Faculty of Bioscience and Biotechnology, Tokyo Institute of Technology, Tokyo, Japan. The constructed YA, YB, and YC/pcDNA3 vectors (restriction enzymes: BamHI [from *Bacillus amylol*i and NdeI], which are commercially flagged was sequenced. Then, 5×10^5 mouse stromal cells (ST2: ODF-expressing cells in mouse) were spread in a 10 cm Petri dish. After 24 hours, the cells were transfected using 60 ml Lipid Effectene (QIAGEN, Tokyo, Japan), medium (1 x MEM- α , 10% fetal bovine serum) was changed before adding transfection reagent to cells and changed every night. After 48 hours, the cells had cross-linked.

Formaldehyde cross-linking.³⁻⁷ For in vitro cross-linking, 10 ml of cross-link solution type 1 (11% formaldehyde, 50 mM Hepes (pH 8), 100 mM sodium chloride (NaCl), 1 mM ethylene diamine tetraacetic acid (EDTA), 0.5 mM egtazic acid (EGTA) (made fresh for each use), and HCHO (from 37% stock containing 10% methanol, added just prior to use), was added to a 10 cm dish (medium was removed in advance), after 15 minutes of gentle shaking, reaction was stopped using 1 ml stop solution (25 M glycine, 10 ml Tris-HCl [pH 8] made fresh for each use) for 5 minutes. For checking optimal cross-linking condition, 1 ml of the cross-linking solution type 1 was added to the Petri dish containing a medium with an amount of 0.7%, 1.3%, and 1.8% formaldehyde. Selected times were 3 minutes, 8 minutes, 30 minutes, (in the case of NFY-A, 40 minutes). After adding 1 ml stop solution, the dishes were shaken gently for 5 minutes until the stop solution was spread throughout. Cross-linking was also carried out with 0.5%, 1%, and 2% formaldehyde, within 45 minutes, using a total volume of 20 ml buffers, including 10 ml cross-link solution type 2 (0.5%, 1%, 2%, formaldehyde respectively for 0.5% cross-linking, 1% cross linking and 2% cross linking, and 50 mM Hepes, 100 mM NaCl, 1mM EDTA, 0.5 mM EGTA, diluted with water to a total volume of 10 ml), and 10 ml medium. The selected time was 45 minutes, overnight, and room temperature at 37°C. For endogenous linking, 5×10^5 cells were spread in a 10 cm dish, and after 72 hours, the cells were harvested as in the previous protocol, but after separating the

supernatant from the nuclei, the nuclei were suspended with Tergitol-type NP-40, and then cross-linked with a cross-linking solution; 20 ml of cross-linking solution was added to the sample (180 μ l nuclear extracts), then inverted 2-3 times, and incubated for 15 minutes at room temperature. The reaction was stopped by adding 20 μ l stop solution.

Immunoprecipitation.⁸⁻¹⁰ For nuclei extract preparation, the medium was removed, and cells were washed 2 times with 2 ml phosphate buffered saline. The cell was harvested and transferred to a tube, and centrifuged at 8000 rpm at 4°C for one minute. Then, the supernatant was removed, 1 ml hypotonic buffer (10 mM Hepes [pH 7.9], 50 mM potassium chloride [KCl], 0.5% NP-40) was added, and the product was suspended gently 30 times. This separated the nuclei from the cytoplasm, then the tube was incubated 20 minutes on ice, homogenized with homogenizer (20 strokes), centrifuged 5000 rpm at 4°C for one minute, and the pelleted nuclei were suspended in 400 μ l liter sodium dodecyl sulfate buffer (SDS) (50 mM Tris [pH 8], 1% SDS, 10 mM EDTA, 1% NP-40 replaced with 1% SDS for cross-linking, sonicated for 10 minutes (cycle 50%, limit 5), centrifuged at 15000 rpm at 4°C for 10 minutes, and checked by agarose gel (length of fragment should be 500-1000 base pair [bp], and it should have a similar size in all samples as fragment length has an effect on the efficiency of immunoprecipitation). Here, a fragment length of 1000 bp was chosen for all samples. Nuclear extract was prepared and stored at -80°C. The sample was rotated at 4°C overnight (O/N), 5 rpm (2 hours is sufficient), then centrifuged at 8000 rpm at 4°C for one minute, washed 2 times with 500 μ l lysis buffer, (50 mM Hepes, 500 mM NaCl, 1 mM EDTA), 1% Triton, 0.1% Na deoxycholate, 1 ml phenylmethanesulfonyl fluoride, 10 μ g/ml aprotinin, 1 μ g/ml leupeptin, 1 μ g/ml pepstatin) 5 times with washing buffer containing lithium chloride (LiCl) (10 mM Tris [pH 8] 0.5 M LiCl, 0.5% NP-40, 0.5% sodium deoxy, 1 mM EDTA), and one time with 1 x TE (10 mM Tris-HCl [pH 8], and 1 mM EDTA [pH 8]). The flag peptide (0.5 μ g/ μ l) was mixed with 30 μ l immunoprecipitation (IP) buffer as the elution buffer. Elution was performed 2 times for each sample. We collected the supernatant very rapidly after centrifuging. The existence of the antibody or beads in the sample decreases the quality of data, especially in cross-linking experiments, as each band in a lane represents one interactor. The presence of beads and antibodies containing nonspecific proteins would have caused an error in the interpretation of data. After combining elution 1 and elution 2, we centrifuged at 10000 rpm for 5 minutes to remove contamination from the sample, and remove the supernatant (avoiding the bottom of the sample). Parafilm was used to seal the tube (otherwise the sample might evaporate), then

reverse cross-linking was performed for 6 hours at 65°C. This time was sufficient for cross-linking dissociation. A longer incubation time is not recommended as an increase in temperature causes a decrease in pH, and in low pH, protein will be degraded, especially low-concentration protein (the volume of sample is not important for determining reversing time). Likewise, increasing the salt in the elution buffer (here, IP buffer) decreases protein degradation, but too much salt causes difficulty in protein dissociation. Then, 9 μ l of the sample was mixed by a mixer for 5 minutes after heat shock (5 minutes at 98°C), and run in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by Western blotting, to detect recovery of protein after IP. A 3 μ l of NFY A sample, and 4 ml of NFY B and NFY C samples were used for silver staining using 5 μ l "4 x dye," and diluted to 20 μ l with IP buffer. The sample for Western blotting was run in SDS-PAGE (200 V, 45 minutes). As the amount of protein of interest is often below the limit of detection by simple Western blotting, total proteins can be labeled *in vivo* with S³⁵, which improves the sensitivity of detection of specific chromatin components after immunoprecipitation. Then, protein bands in SDS-PAGE were transferred to a membrane paper by transfer in 80 V in 90 minutes, and using transfer buffer (20% methanol, 192 mM glycine, 25 mM Tris [pH 8] diluted to 1000 ml). The membrane was blocked by 5% skim milk for one hour, washed with iced phosphate buffer solution 2 times x 5 minutes, then α -flag antibody was diluted 1/1000 (α -VDR 1/300, α -YC and α -YA 1/100, α -TRAP220 1/100) by 5% skim milk in 0.02% sodium azide in Tris-buffered saline Tween-20 (TBST). The blot was incubated with the antibody overnight, then the antibody was removed, and the blot was washed with PBS 4 times x 5 min, and the secondary antibody was determined based on the first antibody (α -mouse immunoglobulin for α -flag antibody, α -rabbit Ig for VDR, α -sheep Ig for YA, YB, and YC), diluted with TBS-T 1000 times, and the blot was shaken for 1 hour, and washed 4 x 5 minutes by PBS. Finally, the blot was incubated with mixture reagent for one minute, and detected by the image master VDCL (Amersham Pharmacia Biotech, Uppsala, Sweden). A dual-color (Bio-Rad, London, England) was used as a marker. The sample (1 μ l for fuse binding protein (FBP2), 3 μ l for NFY A, 4 μ l for NFY B and NFY C) was mixed with 5 μ l of 4 x dye, and diluted to 20 μ l by IP buffer, after subsequent heat shock, the sample was mixed well then applied to SDS-PAGE, and 2D silver stain IDAII CHI, (Daiichi Pure Chemical Co., Tokyo, Japan), and data were prepared. Exposure time was 10 minutes. The SDS broad range marker (Bio Rad, London, England) was diluted 1/20 with 1 x sample buffer before use. After finding optimal conditions for cross-linking, the interaction between the NFYs complex and ODF promoter was detected

by polymerase chain reaction (PCR). The NFY A was used as a template and the ODF promoter in the coding region of +35 was used as a 3' primer and -105 coding region used as a 5' primer. Data shows the interaction between NFY A, NFY B and NFY C and the ODF promoter, which bind to each other naturally in vivo, so detection of the interaction between YA and ODF shows interaction of all subunits.

Polymerase chain reaction. The following protocol was used for PCR: 10 x 5 µl KOD-plus buffer, 5 µl dNTPs, 5 µl magnesium sulfate, 5 µl (10 pmol) dimethyl sulfoxide, 2.5 µl primer, 3 µl template, and 1 µl KOD plus, diluted to 50 µl with deionized water (dH₂O). The PCR was carried out using hot start protocol as follows: 98°C for one minute, 98°C for 15 seconds - 25 cycles, 60°C for 10 seconds - 25 cycles, 74°C for 2 minutes - 25 cycles, 4°C - cool down.

Results. As shown in Figure 1, cross-linking of FBP2 which was carried out in a total volume of 20 ml (10 ml medium in 10 ml cross-link solution) for 10 samples, has one advantage: Western blotting and silver staining data, when the cross-linking was carried out in a total volume of 20 ml, has better quality compared to when it was carried out in a total volume of 10 ml. A repeat experiment for these proteins and other proteins confirmed this fact that increasing the volume of buffer for cross-linking increases the quality

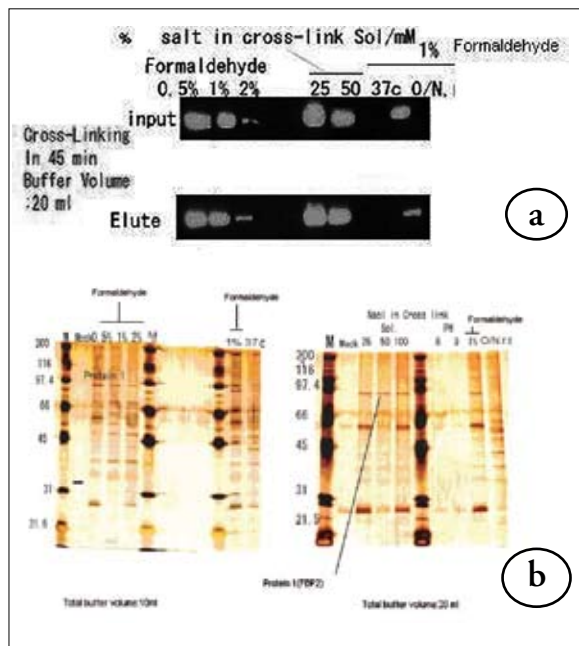


Figure 1 - Fuse-binding protein 2 cross-linking: using 1% formaldehyde is known as an optimal condition for recovery after cross-linking and immunoprecipitation. Increasing total volume of liquid up to 20 ml in 45 minutes, increases quality of data. Cross-linking at 37°C overnight is not recommended. a) Western blotting data, b) Silver staining data.

of data. Meanwhile, optimal condition for cross-linking was detected, using different amounts of formaldehyde (0.7%, 1.3%, 2.4%), different time (3 minutes, 8 minutes, 15 minutes, 40 minutes, O/N), and different amount of NaCl (25 mM, 50 mM) in buffer. In the case of NFY A: 1.3% formaldehyde for 8 minutes, or 1.3% formaldehyde for 15 minutes was chosen as the best condition (Figure 1). For NFY B: 1.3% formaldehyde for 3 minutes, or 0.7% formaldehyde for 15 minutes was chosen as the optimal condition. For NFY C: 1.3% formaldehyde for 15 minutes, or 0.7% formaldehyde for 15 minutes was chosen as an optimal condition (Figure 2). Figure 3a indicates steps in immunoprecipitation: at first, expression vector NFY A or B/C genomic DNA transfected into stromal St-2 cells. After the desired protein was expressed from the

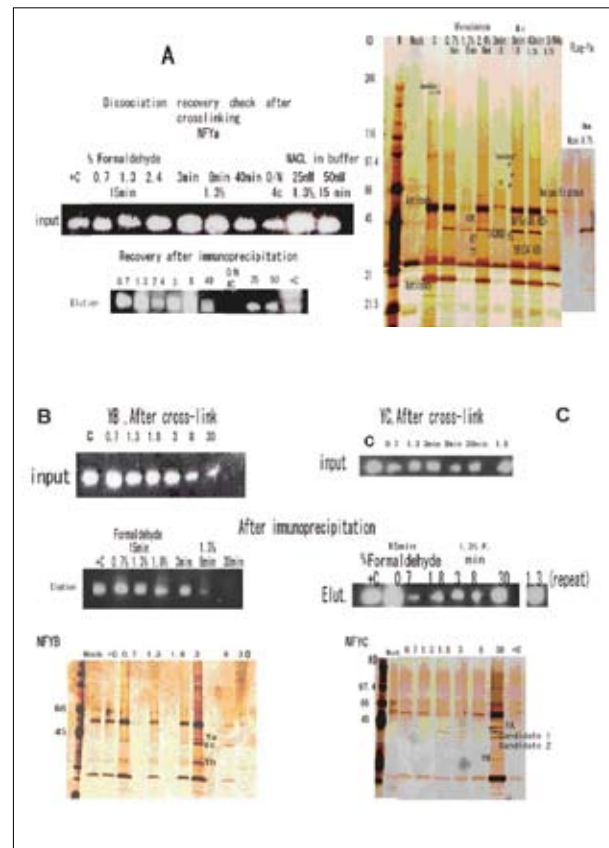


Figure 2 - Recovery after cross-linking and immunoprecipitation were checked. a) for nuclear factor (NFY) A: using 1.3% formaldehyde within 8 minutes and 15 minutes has best recovery. To recognize non-specific proteins, the mock lane was compared with another lane, the existence of the same bands in the mock lane and others, indicate non-specific protein. A comparison between the positive control and lanes indicate optimal conditions for cross-linking. b) for NFY-B: using 1.3% formaldehyde within 3 minutes, and 0.7% formaldehyde in 15 minutes was optimal. c) for NFY-C: using 0.7% and 1.3% formaldehyde for 15 minutes is optimal (considering another silver-staining data, which confirmed 0.7% and 1.3% within 30 minutes).

cell, nuclear extract was prepared using sonication. Then NFY A or B/C precipitated, using anti-flag, anti-body. The cross-linking was reversed, and recovered protein was detected using Western blotting.

Our results also indicate that a mild increase in salt concentration in the elution buffer used in IP increases the efficiency of IP, but too much high salt concentration (around 1000 mM) decreases the amount of recovered protein (Figure 3b). There is a reason for this criteria: when a high amount of solute A (salt) dissolves in a solution (IP buffer), the dissolution rate of solute B will decrease. However, this decrease in recovery does not effect the silver staining data (Figures 3a & 3b). As shown in Figure 4, in vitro cross-linking is recommended, compared to endogenous cross-linking. In Figure 4, protein lanes in vitro cross-linking is clear, but in endogenous cross-

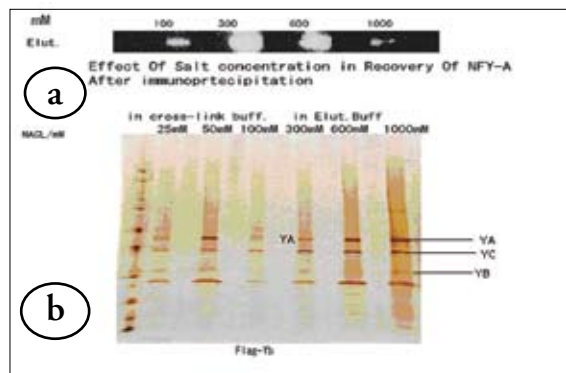


Figure 3 - A mild increase in salt concentration in elution buffer increases recovery after IP, but too high a salt concentration inhibits recovery (1000 mM NaCl in figure a). a) Western blotting data b) Silver staining data.

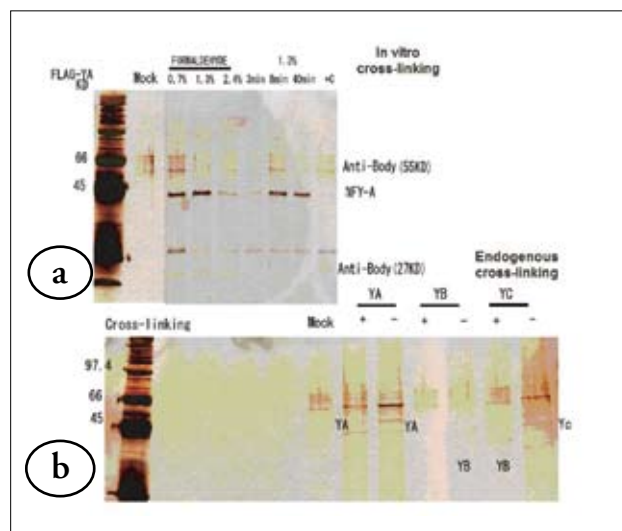


Figure 4 - Comparison between a) in vitro cross-linking and b) endogenous cross-linking. Recovery of protein is very low when cross-linking is endogenous.

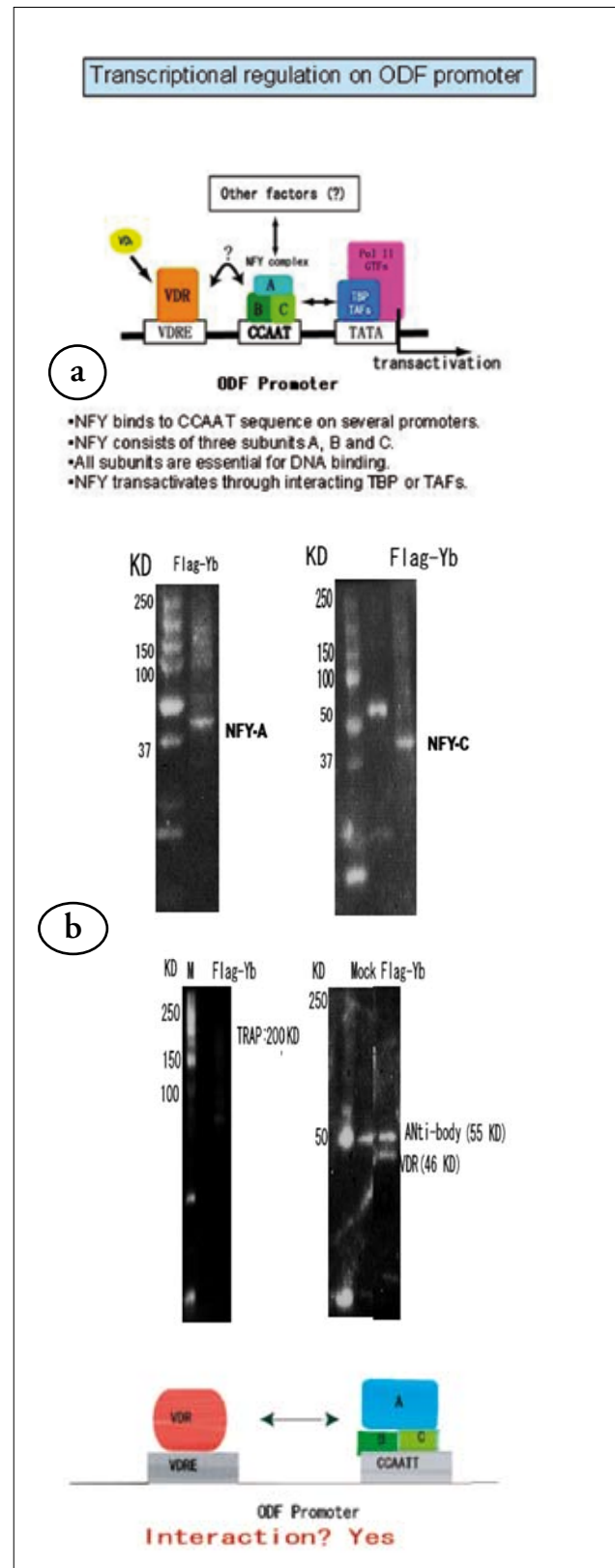


Figure 5 - Transcriptional regulation on osteoclast differentiation factor (ODF) promoter: a) detection of interaction between nuclear factor NFY-A, NFY-C, and NFY-B, b) detection of interaction between NFY's and vitamin D receptor (VDR).

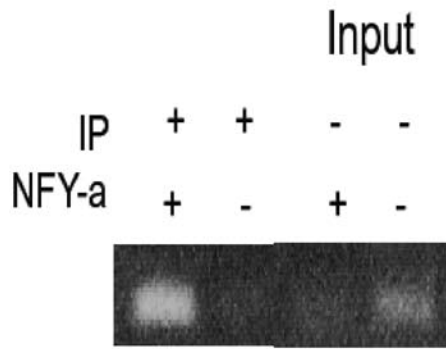


Figure 6 - Detection of interaction between nuclear factor (NFY) (a, b, c) and osteoclast differentiation factor promoter by polymerase chain reaction: lane one shows interaction, lanes 2, 3, and 4 were used as controls.

linking it is quite unclear. Vitro cross-linking has better recovery, as there is a chance to wash the cells with PBS after cross-linking to remove formaldehyde from the cell membrane, and decrease the concentration of formaldehyde in the sample, while with the endogenous method, there is no chance to wash after cross-linking (Figure 4). In Figure 5a, transcriptional regulation on ODF promoter has illustrated the following: 3 elements are involved in ODF expression, VDR, which bind to VDR element, NFY A, B, and C subunits, which bind to CCAAT box, and TBP and TAFs plus Pol II and GTFs, which bind to TATA box (Figure 5a). In Figure 5b, interaction between NFYs and VDR was detected: VDR (46 kDa) band was observed in silver staining data, and then sample detected by α -VDR antibody with Western blotting. The same process was carried out for TRAP 220, however, a band was not observed. Therefore, TRAP 220 is not a TF for ODF (Figures 5a & b). An interaction between NFYs and ODF promoter was detected. The observed DNA band in the first lane of agarose gel shows interaction between NFY subunits and ODF promoter, and address us that NFY subunits role as transcription factor for ODF (Figure 6).

Discussion. The optimal conditions for cross-linking can vary, based on protein concentration in cells, but have a limit from 3 minutes for low concentrations proteins to 15 minutes for (high concentration proteins), also between 0.7% formaldehyde (low concentration protein) to 1.8% formaldehyde (high concentration protein). Factors that affect protein folding like pH, salt, additives, temperature, number of reactive groups, spacer arm length, and buffer volume and composition must also be considered. Compared to previous studies,⁶ the advantage of this study is its focus specifically on optimal cross-linking for NFYs, but in previous studies,⁶ optimal cross-linking was stated generally for all proteins. Meanwhile, cross-linking time and optimal

cross-linking condition based on protein concentration was not discussed in previous studies. Meanwhile, the identification of interaction between NFY subunits themselves and with VDR, are important issues for drug development for osteoporosis. Since osteoporosis is common problem in old-aged women, developing new drugs in this category can be very helpful.

This research has some limitations. Some proteins like NFYs, ODF, and VDR were already identified by mass spectrometry in previous research, but there are other unknown transcription factors for ODF. We hope to identify these proteins in the future using mass spectrometry.

In conclusion, optimal cross-linking conditions vary, based on protein concentration, pH, additives, temperature, number of reactive groups, spacer arm length, and buffer volume and composition.

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