Transcriptional activity of nuclei in multinucleated osteoclasts and its modulation by calcitonin

P. Boissy, F. Saltel, Christine Bouniol, Pierre Jurdic, Irma Machuca-Gayet

0013-7227/02/$15.00/0
Printed in U.S.A.

Endocrinology 143(5):1913–1921

Transcriptional Activity of Nuclei in Multinucleated Osteoclasts and Its Modulation by Calcitonin

PATRICE BOISSY*, FREDERIC SALTEL, CHRISTINE BOUNIOL, PIERRE JURDIC, AND IRMA MACHUCA-GAYET

Laboratoire de Biologie Moléculaire et Cellulaire de l'Ecole Normale Supérieure de Lyon (P.B., F.S., P.J., I.M.-G.), Unité Mixte de Recherche 5665 Centre National de la Recherche Scientifique/Ecole Normale Supérieure, Institut National de la Recherche Agronomique (INRA) 913, 69364 Lyon Cédex 07, France; and Laboratorie BCM (C.B.), INRA Domaine de Vilvert, 78352 Jouy-en-Josas cedex, France

The function of osteoclasts is to digest the calcified bone matrix. Osteoclasts, together with myotubes, are among the rare examples of multinucleated cells found in higher vertebrates, resulting from the fusion of mononucleated progenitors belonging to the monocyte/macrophage lineage. So far, no information is available about function and transcriptional activity of multiple nuclei in osteoclasts. We have used a run-on technique to visualize RNA synthesis in individual nucleus. We provide the first evidence that nuclei of resorbing osteoclasts, isolated from chick embryo long bones, or differentiated *in vitro* from murine spleen cells in presence of RANKL and macrophage-colony stimulating factor, are all transcriptionally active. Nevertheless, if transcriptional activity is the same for all the nuclei within a cell, its level varies between osteoclasts: osteoclasts with highly active nuclei are always associated with resorption pits. We found that global transcription activity of resorbing osteoclasts seeded on calcified matrix is down-regulated after 5-h treatment with calcitonin, which transiently blocks resorption. This effect is reversible because calcitonin removal led to nuclear transcription activation. These results indicate a strong correlation between transcription and resorption. Finally, our data indicate that the resorption pit surface is linearly related to the nuclei number per osteoclast, strongly suggesting that functional advantage of osteoclast multinucleation is to improve resorption efficiency. (*Endocrinology* 143: 1913–1921, 2002)

MULTINUCLEATED CELLS can be observed in transient situation such as the syncytiotrophoblast during embryonic development of mammals or in pathological conditions such as macrophage polykaryons during inflammation as well as after virus infection (1–3). Osteoclasts and myotubes are, in higher vertebrates, two unique examples of permanent functional multinucleated cells under physiological conditions. In most cases, multinucleation results from fusion of mononucleated cells and nuclei in syncytium are usually unable to replicate. However, some syncytia such as the syncytial blastoderm of *Drosophila* during embryonic development or the syncytial endosperm during plant seed development can also be formed by endoploidy, which corresponds to a series of nuclear replication and division without cytokinesis (4, 5).

Unlike myotubes, which are initially formed during embryonic development by fusion of mononucleated myoblasts (6, 7), osteoclasts, which are found at the bone vicinity where they digest the calcified bone matrix, are formed all along adult life by fusion of mononucleated progenitors belonging to monocyte/macrophage lineage. *In vitro*, such precursors can be triggered to osteoclasts if grown in presence of both cytokine macrophage-colony stimulating factor (M-CSF) and RANKL (8, 9). Mature mammalian osteoclasts express some typical markers among them the tartrate-resistant acidic phosphatase (TRAP) isoenzyme and the calcitonin receptor. In presence of calcitonin, bone resorbing osteoclasts transiently retract and stop resorption (2–10). The number of nuclei per osteoclast, which are all gathered in the center of the syncytial cell, isolated from the same bone preparation is extremely variable, from 3 up to 20. Its average number also differs according to animal species with usually only a few in rodents, but more in others species such as cats (11). In addition, in some pathological situations like Paget's disease, each osteoclast can contain more than 100 nuclei (12).

The functional differences between myotubes and osteoclasts, as well as their striking morphological and nuclei distribution differences, suggest that multinucleation does not confer them the same advantages. If the presence of numerous nuclei in myotubes can be easily related to the need of these cells to maintain myofibrils organization along their length, the advantages of multinucleation for osteoclasts are far from evident. An *in vitro* study has shown that a positive correlation could be established between the number of nuclei per osteoclast and the resorption pit size formed in bone (13), supporting indirectly the idea that these multiple nuclei could influence the bone resorption activity. However, nothing is currently known about the transcription activity of nuclei in osteoclasts.

To address this question, we have studied transcriptional activity of nuclei in multinucleated osteoclasts by means of indirect immunolabeling to detect the incorporation of 5′ bromouridine triphosphate (BrUTP) into nascent RNA transcripts (14, 15). In multinucleated bone-resorbing osteoclasts obtained either from chicken long bones or from cultures of murine spleen cells, each nucleus exhibits a transcriptional activity level similar to other nuclei within the same cell.

Abbreviations: BrU, Bromouridine; BrUTP, 5′ bromouridine triphosphate; LSM, leukocyte separation medium; M-CSF, macrophage-colony stimulating factor; TRAP, tartrate-resistant acidic phosphatase; TBS, Tris-buffered saline.

Nevertheless, there was a strong correlation between nuclear transcription activity and bone resorption as clearly indicated by means of the antiresorptive hormone calcitonin, giving a first insight on how the transcriptional machinery could influence the specialized function of osteoclasts.

Materials and Methods

Culture conditions for macrophages

Chicken monocytes were isolated from peripheral blood of 3-month-old SPAFAS chicken according to the procedure described previously (16). Briefly, leukocytes were separated from whole blood by centrifugation through a density gradient of leukocyte separation medium (LSM, ICN Biomedicals, Inc., Aurora, OH). Cells collected from the interface were resuspended in BT88 medium [Dulbecco-derived medium (Biomedia, Bousseus, France, Formula No. DMEMNGS24022988)] supplemented with 5% FCS (Roche Molecular Biochemicals, Mannheim, Germany), 5% chicken serum (Sigma, St. Louis, MO), 10% tryptose (Difco, Detroit, MI) and antibiotics (Biomedia) and allowed to adhere for 1 d at 37 C in 5% CO_2. Nonadherent cells were removed by washing and after 2 d of culture, adherent monocytes differentiate into a homogeneous population of macrophages. Primary macrophages were then trypsinized, numbered and seeded at high density (7.5×10^4 cells/cm^2) on glass coverslips.

Preparation of osteoclasts from chick long bones

Mature osteoclasts were prepared from tibiae of 17-d-old chicken embryos. Briefly, tibiae were dissected out in sterile conditions, and bone marrow was flushed out with a syringe containing BT88 medium. Bone marrow cells were centrifuged through a density gradient of LSM to eliminate erythrocytes. Cells collected from the serum/LSM interface were centrifuged, washed once in BT88 medium and osteoclasts present in cell suspension were counted after trypan blue dye exclusion. Cells were allowed to settle down and adhere on dentin slices (gift from Dr. T. Suda, Tokyo, Japan) for 1 h in BT88 medium at a density of about 150–200 osteoclasts/slice. Nonadherent cells were then removed with several washings and adherent cells, including osteoclasts, were incubated for 2 d in BT88 medium with 10 nM 1,25$(OH)_2$ vitamin D_3 (gift from Leo Pharmaceuticals, Ballerup, Denmark) at 39.5 C in 10% CO_2 to increase the resorption activity.

Isolation and culture of murine spleen cells

Spleens from 6-wk-old mice were mechanically desegregated in αMEM containing 10% FBS (HyClone Laboratories, Inc., Logan, UT) (αMEM/FBS) and the cell suspension was filtered through 100 μm nylon mesh. The leukocyte fraction was separated by centrifugation through a density gradient of LSM. Cells were washed, resuspended in αMEM/FBS containing 30 ng/ml M-CSF (Peprotech, Inc., Rocky Hill, NJ) and 30 ng/ml RANKL (a generous gift from Immunex Corp., Seattle, WA), seeded in culture dishes and maintained at 37 C in 5% CO_2. Medium with factors was replaced every 2 d until formation of multinucleated cells. Osteoclasts were generally used after a week of culture.

The animals were maintained in accordance with the guidelines of Federation of European Laboratory of Animal Science Association and Ministère de l'Éducation Nationale et de la Recherche for the use and care of experimental animals (laboratory approval no. A69035; individual approval no. 006416).

Resorption assay with RANKL-induced osteoclasts

Murine multinucleated cells, differentiated in culture, were washed twice with PBS, incubated with 5 mM EDTA in PBS at 37 C for 20 min and then treated with trypsin at 37 C for an additional 20 min. Floating cells were harvested, pelleted, washed with αMEM/FBS, and plated on dentine slices. After 2 h, dentine slices were incubated in αMEM/FBS with M-CSF and RANKL for 3 d at 37 C in 5% CO_2 (resorption pits are not observed earlier). For resorption inhibition, 10^{-7} M salmon calcitonin (Bachem, Bubendorf, Switzerland) was added at d 2 of cultures. TRAP activity of cells was detected with a kit from Sigma, whereas resorption pits were revealed, after elimination of cells with Triton X-100, by staining dentine slices with toluidine blue.

Resorption activity of osteoclasts assayed on OAAS

Murine osteoclasts obtained as described above, were detached after washing and a 15-min incubation time in presence of 0.25 mM EDTA in PBS and then recovered in αMEM/FBS supplemented with M-CSF and RANKL. They were seeded on a calcium-phosphate thin layer coated 4-well dishes OAAS from OSCOTEC (Torrance, CA). Twenty-four hours later, run-on experiments were subsequently performed, a TRAP assay was completed, and the resorption pits were stained with toluidine blue following cell removal.

Run-on transcription in permeabilized cells

RNA synthesis in multinucleated cells was assayed by BrUTP incorporation in permeabilized cells according to the procedure firstly described by Wansink *et al.* (14). Briefly, adherent cells were washed subsequently first with Tris-buffered saline (TBS) and then with a glycerol buffer (20 mM Tris HCl, pH 7.4; 5 mM $MgCl_2$, 25% glycerol, 0.5 mM EGTA, 0.5 mM phenylmethylsulfonyl fluoride). Cells were then slightly permeabilized with 0.05% Triton X-100 in glycerol buffer for 3 min at room temperature. Cells were washed once in glycerol buffer and run-on transcription was performed in transcription buffer (50 mM Tris-HCl, pH 7.4; 100 mM KCl; 5 mM $MgCl_2$; 25% glycerol; 0.5 mM EGTA; 0.5 mM GTP; 0.5 mM ATP; 0.5 mM CTP; 25 μM *S*-adenosyl-L-methionine, 1 mM phenylmethylsulfonyl fluoride; 1 mM BrUTP; 20 10^{-3} U/ml RNasin (Promega Corp., Madison, WI) for 30 min at room temperature. Then, cells were washed once with TBS containing 5 U/ml RNasin for 5 min and fixed with 2% paraformaldehyde in PBS containing 2% sucrose for 30 min at room temperature. After a rinse in PBS, cells were permeabilized 5 min with 0.2% Triton X-100 in PBS before performing an immunodetection of BrUTP-labeled RNAs. In some conditions, BrUTP incorporation was performed in presence of α-amanitin (Sigma) to block RNA polymerase II activity.

Immunocytochemistry

After fixation and permeabilization, cells were incubated for 20 min with PBS containing 0.6% H_2O_2 to inactivate endogenous peroxidase activity. Preparations were then saturated with 2% BSA in PBS (PBS-BSA) for 1 h at room temperature. To detect bromouridine (BrU) incorporated into nascent RNA, cells were incubated overnight at 4 C with a mouse monoclonal antibody raised against bromodeoxyuridine and also recognizing BrU (MD5300, Caltag Laboratories, Inc., Burlingame, CA) diluted to 1:500 in PBS-BSA. After three washes in PBS, the first antibody was revealed by incubation of cells for 1 h at room temperature with a horseradish peroxidase-conjugated donkey antimouse antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) diluted to 1:500 in PBS-BSA. After three washes of 10 min in PBS and one of 5 min in TBS, peroxidase activity was revealed using 3,3′-diaminobenzidine substrate (Sigma), which forms a black precipitate in the presence of amonium nickel sulfate. Staining reaction was stopped by rinsing coverslips in TBS. Cells were then counterstained with hematoxylin solution (Sigma), washed twice in PBS, and dentine slices were mounted in Glycerol/PBS (9/1) to be observed with a light microscope (AH-2, Olympus, Inc., Rungis, France).

To determine whether the intensity of cytochemical labeling of nuclei can be correlated with their level of transcription, run-on experiments were performed in the presence of increasing concentration of α-amanitin. Nucleus staining of cells on digitized images was analyzed with ImageQuant software [Molecular Dynamics, Inc. (Amersham Biosciences), Orsay, France]. Mean and SD values of the integrated intensity were calculated at each α-amanitin concentration from measures performed on five individual nuclei.

Statistical analysis

All the data are expressed as the mean ± SEM. Statistical evaluation was performed using ANOVA, followed by a *t* test; differences were considered statistically significant at $P < 0.05$.

Analysis of resorption activity

After immunochemical detection of run-on transcription and counterstaining with hematoxylin dye, area of resorption pits formed by osteoclasts containing nuclei highly active in transcription was measured using an image analysis system (Pegase software, 2i system, St Quentin Yvelines, France). Data from 20 osteoclasts were plotted as a function of the number of their nuclei. Statistical analysis of results was performed by using linear regression method and correlation coefficient was then determined.

Results

In situ nuclear staining correlates with the transcriptional activity level in macrophage cultures

Before investigating the transcription activity level in nuclei of mature osteoclasts maintained on dentine slices, we validated the run-on method on macrophage culture to show that the intensity of nuclear staining was proportional to the transcription activity. Briefly, we have incubated living permeated macrophages with BrUTP and immunodetected BrU-labeled RNAs (14, 17). To determine whether the nuclear intensity staining quantitatively reflects the level of BrUTP incorporated during run-on transcription, we examined the effect of increasing concentration of α-amanitin, an inhibitor of RNA polymerase II. Increasing concentration of α-amanitin was correlated with a visual decrease of nuclear staining (Fig. 1, A–E). To quantify this decrease, integrated intensity of staining was measured in five nuclei for each experimental condition (Fig. 1). Statistical analysis shows that mean values of intensity staining per dose of amanitin are significantly different, indicating that this technique is sensitive enough to compare transcription activity level between nuclei.

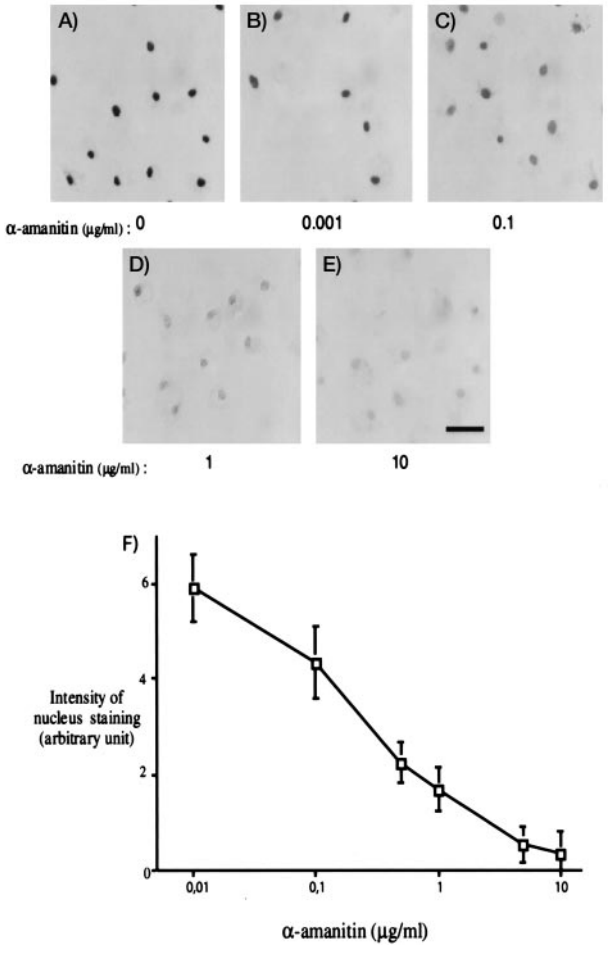

FIG. 1. Intensity of nuclear staining after run-on transcription experiments followed by immunocytochemical detection, correlates with RNA polymerase II activity level. Cultures of macrophages were incubated with increasing concentrations of α-amanitin for 30 min during the BrUTP incorporation step of the run-on. A–E, Micrographs of the resulting immunocychemical staining of nuclei for increasing inhibition of transcription. *Bar*, 40 μm. B, Digitized images of labeled nuclei were analyzed with ImageQuant software and the mean values of the integrated intensity for individual nuclei were calculated (n = 5). F, These values were plotted against the α-amanitin concentration with their respective SD.

Nuclei of resorbing osteoclasts exhibit high transcriptional activity

Using these *in vitro* run-on experiments, we studied next the transcriptional activity of resorbing osteoclasts obtained from long bones of chick embryos and maintained 2 d on dentine slices. Figure 2 shows a series of different bone resorbing osteoclasts (Fig. 2, A and B; C and D; E and F) photographed at different focal planes above resorption cavities, with *arrows* indicating pit limits. In contrast to osteoclasts maintained on coverslips in which all nuclei are in the same focal plane, nuclei of polarized bone resorbing osteoclasts are always located at the center of the syncytium but more dispersed along z-axis with some nuclei found at the resorption lacunae level (Fig. 2, B and D). In both small (Fig. 2, A and B) and large osteoclasts (Fig. 2, C and D), all nuclei showed *black staining* reflecting their immunocytochemical labeling after run-on transcription. It has to be noted that stromal cells surrounding osteoclasts also exhibited a staining of their nuclei but weaker than osteoclast nuclei (Fig. 2A, *arrowheads*). We also performed negative controls to distinguish specific labeling from potential background due to cytochemical procedure. In absence of BrUTP, nuclei counterstained by hematoxylin, were not labeled (Fig. 2E), whereas the addition of α-amanitin at 20 μg/ml during run-on experiments allowed detection of ribosomal RNA only, appearing as a black dot in the nucleolus (Fig. 2F, *arrowhead*). This result indicates that in mature osteoclasts able to resorb bone matrix, all nuclei are transcriptionally active.

We have observed that, whereas nuclei within the same cell were always equivalently labeled, the staining intensity could vary between osteoclasts on the same dentine slice. As illustrated in Fig. 3, we found cells presenting a strong nuclear staining as described above (*arrowhead*), and osteoclasts (cells indicated by *arrows*), exhibiting a *gray staining* equivalent to or lower than, that of stromal cells surrounding them. These variations could not result from technical artifacts because osteoclasts exhibiting dark or faint staining could be found in close vicinity on dentine slices (Fig. 3, C and E, *arrowheads vs. arrows*). More interestingly, our observations revealed that osteoclasts with strongly labeled nuclei (Fig. 3, C and E, *arrowheads*) were always associated with a resorption pit (Fig. 3, D and F, *asterisks*), whereas osteoclasts with weakly stained nuclei (Fig. 3, A and C and E, *arrows*) were

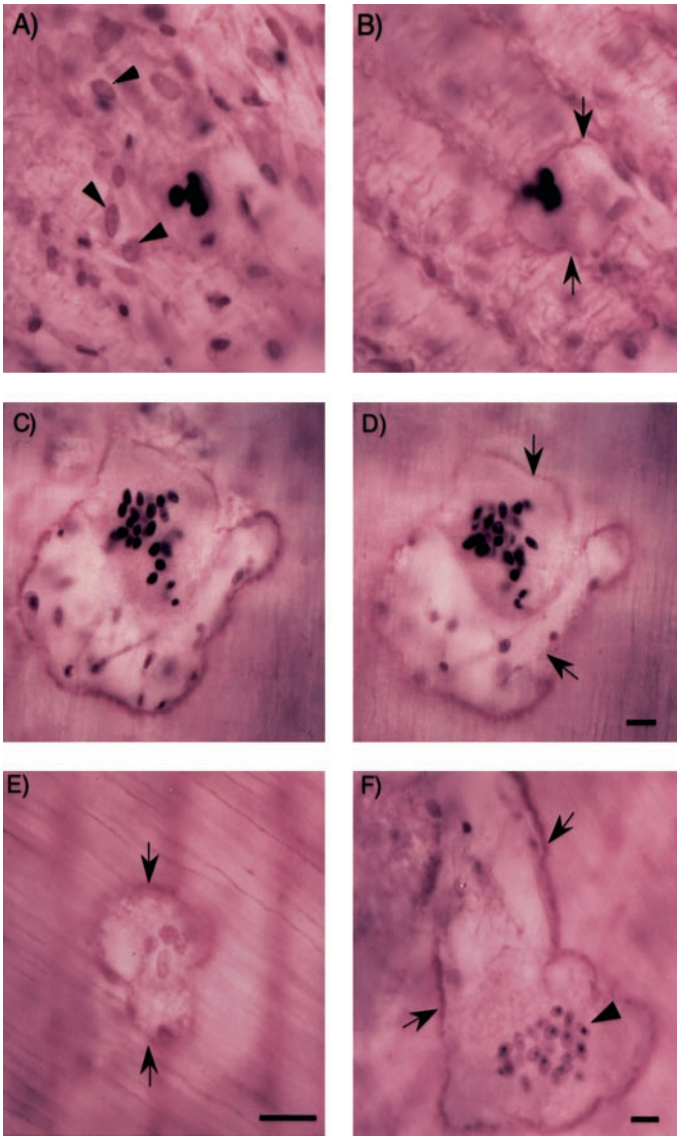

FIG. 2. Transcriptional activity of nuclei in mature osteoclasts resorbing dentine slices. Osteoclasts prepared from long bones of 17-d-old chick embryos, were maintained for 2 d on dentine slices before performing run-on transcription assays. BrU-labeled RNAs are revealed by immunocytochemistry and cells were counterstained with hematoxylin. A small (A and B) and a large osteoclast (C and D) resorbing dentine matrix have been photographed at different focal planes from above to inside the resorption pit. Note that weakly stained nuclei from stromal cells (*arrowheads*) can be detected in the vicinity of darkly stained osteoclasts. As negative controls, run-on experiments were also performed in absence of BrUTP (E) or in presence of 20 μg/ml α-amanitin (F). *Arrows* indicate the borders of the resorption pits. *Bar*, 25 μm.

either nonresorbing (Fig. 3, B and D, *circles*) or found associated with small resorption pits (Fig. 3F, *asterisk*). Counting of the labeled *vs.* unlabeled osteoclasts from one representative experiment out of six showed indeed that 95% of strongly labeled osteoclasts were associated with a resorption pit.

Our results clearly show that l) transcriptional activity is similar between nuclei of the same cell, but varies within the osteoclast population; 2) osteoclasts presenting a high transcriptional activity are always resorbing cells, whereas nonresorbing osteoclasts exhibit always a lower nuclear transcriptional activity. Altogether, these results suggested that resorption activity could be correlated to a high transcriptional activity of all nuclei in a single cell.

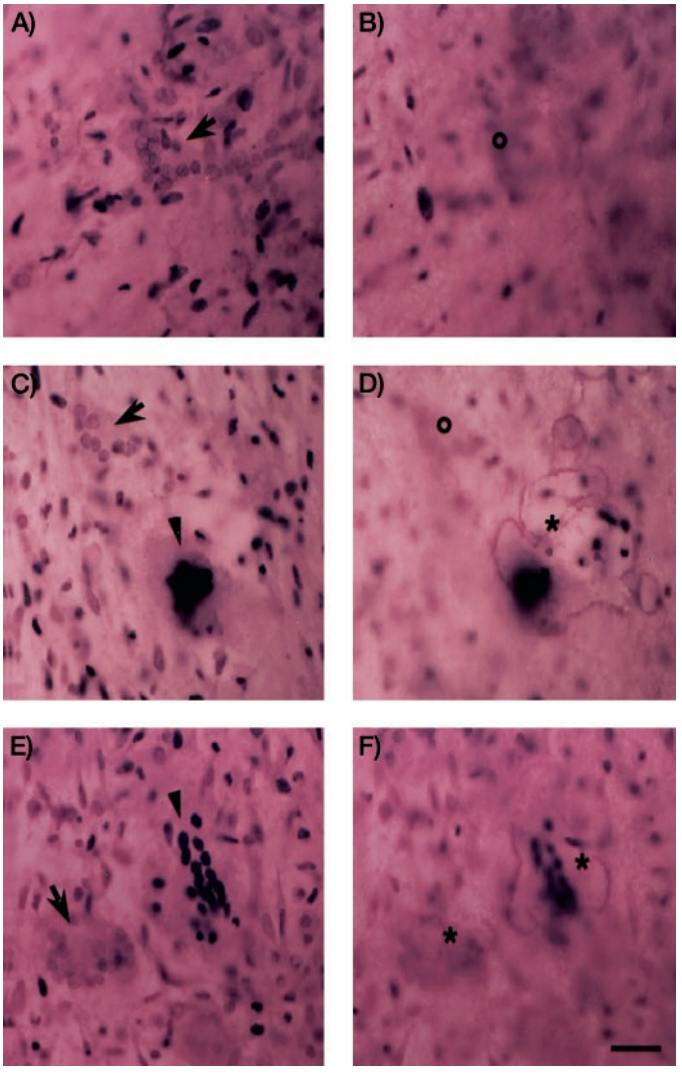

FIG. 3. Transcriptional activity detected in osteoclast nuclei can vary between osteoclasts. Photographs of several osteoclasts have been taken either at the cell level (A, C, E) or at the dentine level (B, D, F) to show resorption pit when present (D, F, *asterisk*) or not (B, D, *circle*). Two types of osteoclasts are observed with either intensively labeled nuclei (C, E, *arrowhead*) or weakly stained (A, C, E, *arrows*) as surrounding stromal cells. Note that within individual osteoclasts all nuclei are stained with the same intensity. Osteoclasts with strongly labeled nuclei (C, E, *arrowhead*) are always associated with a resorption pit (D, F, *asterisks*), whereas osteoclasts with weakly stained nuclei (A, C, E, *arrow*) could be found either associated (F, *asterisk*) or not (B, D, *circles*) with a resorption pit. *Bar*, 50 μm.

Calcitonin response by in vitro generated murine osteoclasts

To further prove that resorption and transcriptional activity are linked, we decided to use the property of calcitonin to transiently induce osteoclast retraction and to inhibit resorption. We performed *in situ* run-on analysis on murine osteoclasts maintained 3 d on dentine slices because the

presence of calcitonin receptor on avian osteoclasts is still debated, but also to rule out the possibility that our observations were restricted to chick osteoclasts. Using osteoclasts derived in culture from spleen cells cultivated in the presence of M-CSF (30 ng/ml) and RANKL (30 ng/ml) we were able to confirm that, like chicken osteoclasts, nuclei from murine osteoclasts are intensely stained when they are actively resorbing dentine (data not shown).

Murine osteoclasts in culture, prepared from murine spleen cells grown in the presence of M-CSF (30 ng/ml) and RANKL (30 ng/ml), were detached and seeded on dentine slices for 2 d before calcitonin (10^{-7} M) was added for another 24 h. Under these conditions, in absence of calcitonin, TRAP-positive osteoclasts were spread out (Fig. 4A) and had actively started to resorb dentine (Fig. 4A, *inset*), whereas 24 h after calcitonin addition, osteoclasts were still TRAP positive, but had retracted (Fig. 4B) as described by others and had poorly resorbed dentin (Fig. 4B, *inset*). We confirmed that, in our conditions, calcitonin is an efficient inhibitor of osteoclast resorption and that this morphological change is reversible. Indeed, if 5 h of calcitonin treatment was sufficient to induce a strong retraction of murine osteoclasts, they were seen to be completely spread out 12 h later after hormone withdrawal (data not shown).

However, adding calcitonin treatment on the top of run-on experiments rendered rather difficult the observation of labeled nuclei when dentine slices were used. The intensive retraction of osteoclasts upon calcitonin treatment, as well as the heterogeneity of dentine slices, and the matrix lack of transparency were detrimental for our purpose. We turned our interest toward calcium-phosphate coated plates (OAAS); 24 h after the osteoclasts attached to the mineral matrix, run-on was performed following a 5-h treatment with calcitonin 10^{-7} M or not. Figure 4, C and E, shows actively resorbing osteoclasts under the control conditions: they exhibit intense labeled nuclei, and are associated with a resorption pit appearing on one side of the cell membrane border (*arrows*). In the presence of calcitonin, treated osteoclasts exhibited absolutely no labeled nuclei (Fig. 4D). TRAP assay was necessary to identify the cells at the matrix surface and further staining with hematoxylin was performed to detect their nuclei (Fig. 4F). The calcified matrix resorption was monitored by toluidine blue staining after cell removal, and the resorption pits clearly appeared as white dots (Fig. 4, C and D, *insets*). The absence of nuclei labeling of the calcitonin treated (Fig. 4D), *vs.* the cognate control (Fig. 4C), was accompanied by a scarce number of pits (Fig. 4D, *inset*), indicating a dramatic impairment of resorption as well as transcription, strongly associating these two major biological osteoclast activities.

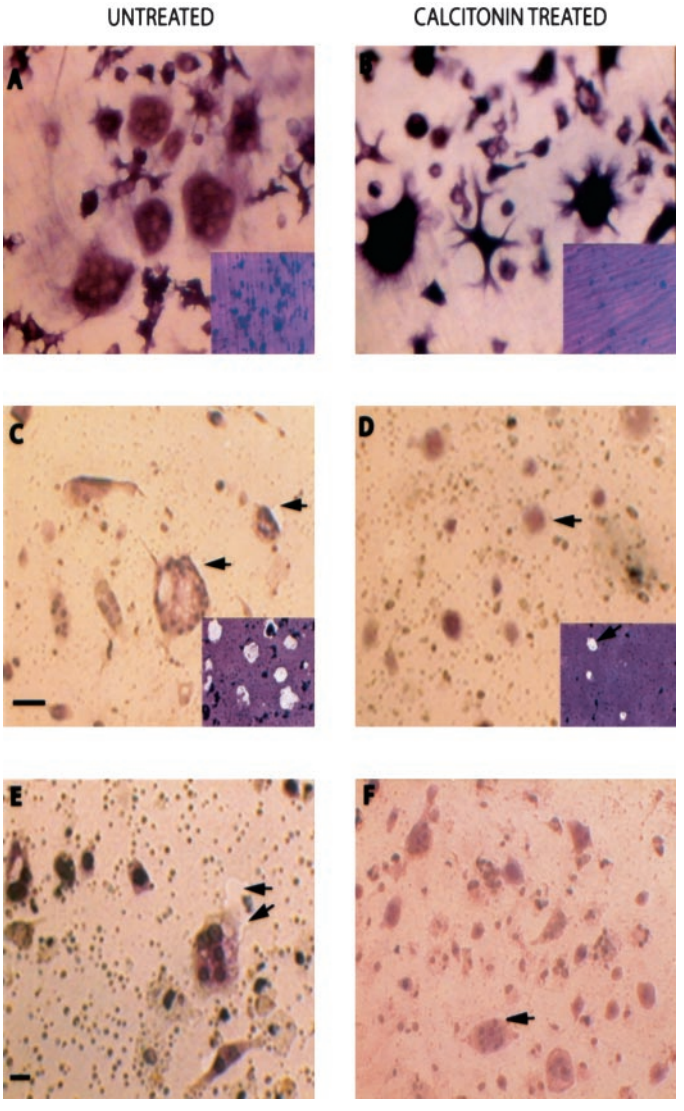

FIG. 4. Transcriptional activity of murine osteoclasts prepared *in vitro*. Calcitonin inhibits dentine resorption and induces a reversible retraction of osteoclasts. Calcitonin (10^{-7} M) was added to spleen cell-derived osteoclasts previously maintained 2 d on dentine slices in presence of M-CSF (30 ng/ml) and RANKL (30 ng/ml). Twenty-four hours after calcitonin addition, TRAP activity was first tested and cells removed from the dentine slice to evaluate resorption after toluidine blue staining (A and B, *insets*). Untreated osteoclasts were spread (A) and had formed numerous resorption pits on dentine (A, *inset*), whereas calcitonin-treated osteoclasts were retracted (B) and had a weak resorption activity (B, *inset*). *Bar*, 100 μm. Inhibition of calcitonin-dependent resorption correlates with transcriptional down-regulation (C–F). Spleen cell-derived osteoclasts were seeded on calcium-phosphate thin layer-coated culture dishes (OAAS). Twenty-four hours later, a run-on experiment was performed either on 10^{-7} M calcitonin treated cells (D–F) or untreated (C–E). *Arrowheads* in C and D are pointing to the resorption, which appeared as *white dots* after cell removal and toluidine blue staining (C and D, *insets*). *Bar*, 40 μm. TRAP activity test (E, untreated) and hematoxylin staining were completed to visualize respectively the cells and the nuclei in F (calcitonin treated).

Reversible calcitonin-mediated cell retraction correlates with transcriptional down-regulation in murine osteoclasts

To examine more precisely the calcitonin effect on transcription in murine osteoclasts, the same experiment was repeated on plastic Petri dishes. Sensitivity of cells to calcitonin treatment was estimated according to their morphology. In the absence of the hormone, osteoclasts were spread out and exhibited a strong staining of their nuclei (Fig. 5A, *arrow*). In contrast, most of them were retracted after treatment with calcitonin and their nuclei were not stained indicating an undetectable level of transcription (Fig. 5B, *arrow*). It must be noted that the inhibitory effect of calcitonin

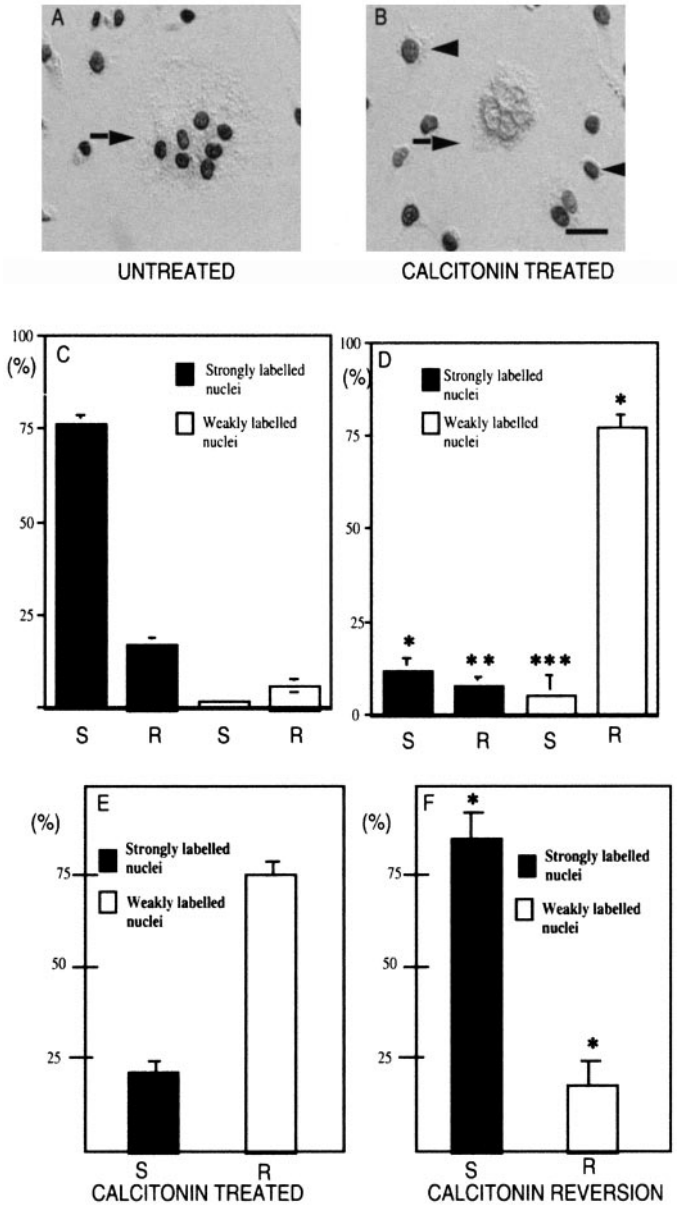

FIG. 5. Calcitonin specifically inhibits transcription in murine osteoclasts. Spleen cell-derived osteoclasts were either untreated (A) or treated (B) for 5 h with calcitonin (10^{-7} M) before carrying out run-on. Osteoclasts nuclei (A, *arrows*) exhibit either high activity in absence of calcitonin or undetectable activity after 5 h of calcitonin treatment (B). Note that in presence of calcitonin, surrounding mononucleated cells are all transcriptionally active (B, *arrowheads*). Statistical distribution of osteoclasts according to both morphology (S, spread; R, retracted) and nuclear transcriptional activity (*black stained* or unstained nuclei) in absence (C) or in presence of calcitonin (D). Statistical distribution of osteoclasts according to both morphology (S, spread; R, retracted) and nuclear transcriptional activity (*black stained* or unstained nuclei) after 5 h of treatment with calcitonin (E), then followed by 20 h of incubation in the absence of hormone (B). Cells were washed twice with culture medium at the end of the 5-h incubation with calcitonin. The results of the *t* test analyzing the differences between the control and the treated group are shown in the figures as *asterisks*. *Asterisks* represent a *P* value for paired data: *, $P < 0.001$; **, $P < 0.05$; ***, $P > 0.05$.

on transcription was restricted to osteoclasts since the surrounding mononucleated cells all exhibited an intense nuclear staining indicating ongoing transcription (Fig. 5B, *arrowheads*). Statistical analysis of osteoclast distribution according to both morphology and nuclear transcriptional activity has revealed that in the absence of calcitonin, 75% of osteoclasts were spread out with intense nuclear staining (Fig. 5C), whereas after 5 h of calcitonin treatment this percentage was down to 10%. On the other hand in the presence of calcitonin, 75% of osteoclasts were retracted with unlabelled nuclei (Fig. 5D).

If transcription arrest is at least one mean by which calcitonin inhibits bone resorption under physiological conditions, this process should be reversible. We have treated the osteoclasts with calcitonin during 5 h (Fig. 5E), followed by 20 h of incubation without the hormone (Fig. 5F). We observed that over 75% of the osteoclasts recovered their flattened shape and a high labeling of their nuclei. These results clearly indicate that in osteoclasts, transcriptional activity is strongly associated with their ability to resorb calcified matrix.

Bone resorption is linearly correlated with the number of nuclei in osteoclast

Because transcriptional activity of all nuclei is high in resorbing osteoclasts and low in resting or calcitonin inhibited osteoclasts, one would expect that the number of nuclei per cell would influence resorption activity of osteoclasts. Hence, we further examined, simultaneously, resorption and transcription activities of osteoclats as a function of their number of nuclei. After 2 d on dentine slices, osteoclasts containing variable number of nuclei are able to form resorption lacuna of different sizes. Cells with few nuclei were associated with small resorption pits (Fig. 6, A and C), whereas those containing numerous nuclei were generally found on top of larger pits (Fig. 6, B and D). As these observations suggested a potential link between these two parameters, we checked more carefully osteoclasts exhibiting a high transcriptional activity and measured for each of them both the area of their resorption pit and their content in nuclei. When data obtained from 20 osteoclasts containing a wide range of nuclei (from 3 to 75) were plotted (Fig. 6E), the area of dentine resorbed per osteoclast was found to be linearly correlated with a high correlation coefficient ($r^2 = 0.967$) with its nuclei number.

Discussion

Myotubes, one of the best-known examples of multinucleated cells in vertebrates, are formed during embryonic development by fusion of mononucleated myoblasts (6, 7). After birth, the subsequent muscle growth depends on recruitment of more myoblasts into the existing multinucleated cells. Using an *in vitro* model of myotube differentiation, different studies have shown that some specific genes exhibit a differential expression in nuclei distributed all along the fiber (18, 19). For example, mRNA coding for the subunits of acetylcholine receptor, a protein localized in the postsynaptic membrane of the neuromuscular junction, is not uniformly distributed throughout the fiber, but is concentrated near

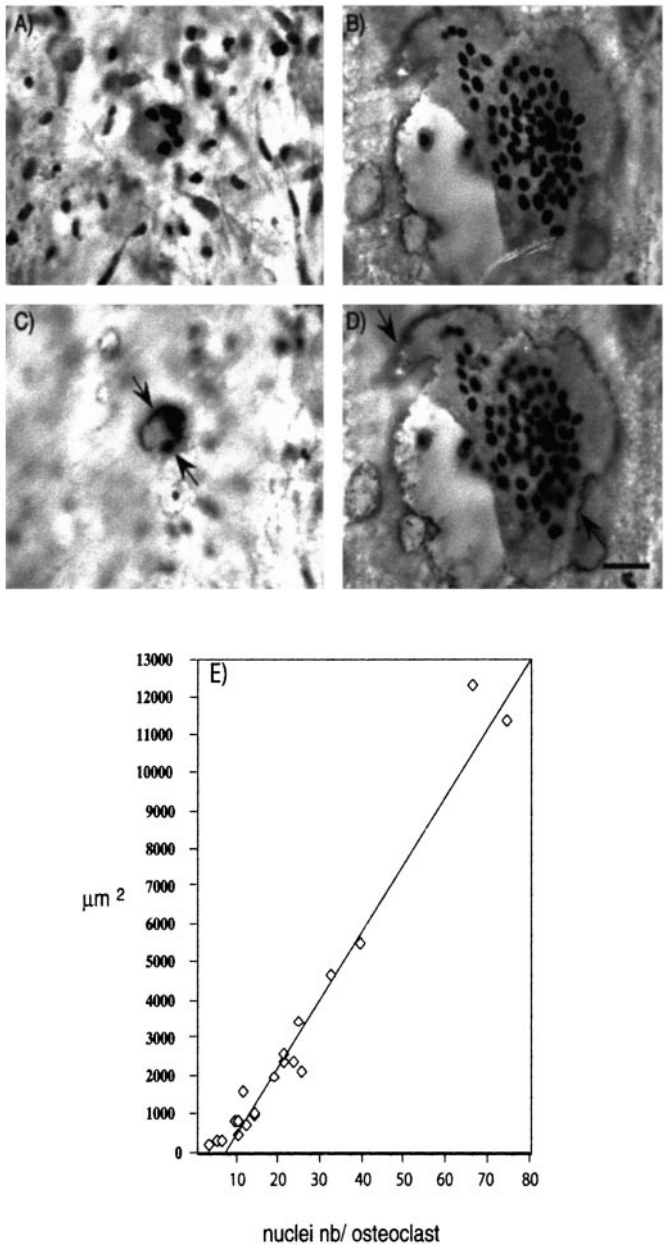

FIG. 6. Osteoclast resorption activity correlates with their number of nuclei. Two osteoclasts exhibiting intensively stained nuclei after run-on experiments are shown associated to their resorption pit. One osteoclast with 6 nuclei associated with a small pit (A and C, respectively) and one with around 75 nuclei with a very large lacunae (B and D, respectively). *Bar*, 40 μm. Resorption pit areas of 20 osteoclasts were measured using an imaging analysis system and plotted against the number of nuclei per osteoclast (E). Correlation coefficient of the linear regression: $r^2 = 0.967$.

nuclei at the endplate. These observations suggest that individual nuclei within skeletal muscle fibers are compartmentalized and may be autonomous with respect to the transcription of at least some specific genes encoding for membrane proteins (20). In contrast, osteoclasts are formed by fusion of mononucleated progenitors belonging to monocyte/macrophage lineage (8, 21). They exhibit a circular shape and although multinucleated, are very mobile (22). When they initiate bone resorption, unpolarized resting or migrating osteoclasts are rapidly transformed into highly polarized cells with several different plasma membrane domains (23).

Exploitation of a sensitive technique allowing one to visualize *in situ* newly synthesized transcripts in living cells (14) has enabled us to investigate the transcriptional activity of nuclei in multinucleated osteoclasts. After cell permeation, BrUTP is incorporated into nascent transcripts during run-on transcription, and BrU-labeled RNAs are then visualized by immunodetection technique and are exclusively found in nucleus. Their transcription is mediated by RNA polymerase II because inhibition by α-amanitin restricts RNA labeling to the nucleolus, mostly represented by rRNA. The retention of BrU-labeled RNAs in nucleus is probably due to a defect in processing of primary transcripts because it has been shown that the presence of BrUTP in RNAs disturbs the splicing process that precedes exportation of mRNAs into cytoplasm (24). Interestingly, this run-on transcription assay allows semiquantitative measures because gradual inhibition of RNA polymerase II activity correlates with gradual decrease in nuclear staining after immunocytochemical detection (Fig. 1). From these results, we can conclude that, within the same experiment, the intensity of the diffuse nuclear labeling directly reflects the level of RNA polymerase II activity.

Electron microscopy studies have shown that nuclei within osteoclasts in bone exhibit different shapes: some nuclei are round and euchromatic, whereas others are irregular in shape and more heterochromatic (25). As chromatin morphology generally reflects the activity of the nucleus, these observations suggested that transcriptional activity between nuclei within the osteoclast could be different. In our experiments, we have demonstrated that a functional mature osteoclast has all its nuclei transcriptionally active, each exhibiting a similar activity (Fig. 2, A–C). Interestingly, we have also shown that the area of resorption formed by each osteoclast can be linearly correlated to its number of nuclei and that the number of nuclei is not a limiting factor for the resorption activity (Fig. 6). Based on our results, we propose that osteoclast multinucleation offers functional advantages not to initiate but mainly to improve resorption activity. However, the question as to whether their implication in resorption activity itself is direct or not remains open. One possibility could be that the function of multiple nuclei for osteoclast is mainly to supply enough housekeeping gene products to maintain a large-sized cell which could be considered as a functional advantage for osteoclasts. Just before resorption, polarized osteoclasts elaborate a tight sealing zone between their ruffled membrane and bone, delimiting an isolated compartment in which they will release protons and proteases involved in bone matrix degradation (23). In this way, large osteoclasts can cover a larger area of bone and consequently form large resorption pits. This hypothesis is supported by data from others showing a linear correlation between the volume of chicken or rat osteoclasts and their number of nucleus (26), and by a study showing that multinucleation enhances macrophage-mediated bone resorption. Rat polykaryons in culture, derived from peritoneal stimulated and purified macrophages, showed an increased

ability to bind and digest isotopic-labeled bone particles compared with mononuclear cells (27).

Our work has also revealed that all nuclei within the same osteoclast always exhibit a similar global transcriptional activity whose level can vary between each osteoclast. The results showed that osteoclasts with a high transcriptional activity are resorbing, whereas nonresorbing osteoclasts have a low transcriptional activity (Fig. 3A). In a pioneer work, Zheng *et al.* (28), using titrated uridine autoradiography to examine RNA synthesis and calcitonin responses in rat osteoclasts, had also shown an isotopic labeling of all nuclei within an osteoclast. However, they saw an inverse correlation between the nuclear staining intensity and the nuclei number: the 2–3 nuclei containing osteoclasts exhibited a stronger staining per nucleus than 10 nuclei osteoclasts. We observed some hetereogenity among the labeled osteoclast when they were purified from bone, but this was regardless of the nuclei number and probably due to a different cell state. We found, indeed, a minor group of resorbing osteoclasts, showing a weak nuclei staining but yet associated with a small pit. These variations of the transcriptional level among osteoclasts is especially found when chick osteoclasts were isolated from bone and seeded on dentine slices along with contaminating stromal cells, in contrast to the murine ones generated by *in vitro* differentiation without any other interfering cell type. The global transcriptional activity is determined by the balance between resting and active osteoclasts and by the fact that all nuclei of each syncytium are simultaneously regulated. This conclusion is supported by the fact that the nuclei in osteoclasts are all located in the center of the syncytium in contrast to those of myotubes, which are scattered along the length of the cell. The gathering of nuclei in the same cytoplasmic domain suggests that signaling induced by external factors would affect similarly gene transcription in all clustered nuclei. In this case, an osteoclast receiving external factors, which influence its resorption activity, could efficiently commit, or not, all its nuclei in bone degradation. In that sense, it is probably a much more efficient way to exert a tight regulation on gene expression in syncitium rather than having juxtaposed mononucleated cells. The study of the expression of specific gene (*i.e.* encoding carbonic anhydrase II or cathepsin K) examined by *in situ* hybridization with intronic probes could bring a definitive answer.

As described by others (29), we have observed that calcitonin induces osteoclast retraction and drastically inhibits pit formation on dentin slices. Moreover, 5 h after addition of calcitonin on osteoclasts, no global transcription activity could be detected in osteoclast nuclei. This effect of calcitonin is osteoclast specific because a normal transcriptional activity can be visualized in surrounding mononucleated cells. The calcitonin-treated osteoclasts with undetectable transcription activity are not dying cells, as the effect of calcitonin is transient and completely reverted in about 12 h after withdrawal. Zheng *et al.* (28) have also shown that calcitonin reduced markedly the nuclear titrated uridine uptake in binucleated osteoclast but seemed to have a milder effect on multinucleated cells. Their results suggest that, in this study, the osteoclasts containing an elevated number of nuclei showed a reduced responsiveness to the labeling method and to the calcitonin. Our results fit with an earlier observation that osteopontin transcript is down-regulated in isolated rabbit osteoclasts 3 h after calcitonin addition (30). A calcitonin-induced down-regulation of calcitonin receptor has also been described, although it is not clear whether it is due to an inhibition of transcription or a reduced life span of the transcripts (for review, see Ref. 10). However, because resorption of calcified matrix is a slow process, our results do not permit one to conclude whether the down-regulation of transcription is directly involved in resorption arrest or if it results from a secondary effect of calcitonin and yet the question remains open if another bone resorption inhibitor could act through a transcriptional inhibition. Further studies will be necessary to unravel the precise molecular mechanisms linking regulation of osteoclast transcription and resorption activity.

Acknowledgments

Received October 11, 2001. January 31, 2002.

Address all correspondence and requests for reprints to: Irma Machuca-Gayet, Laboratoire de Biologie Moléculaire et Cellulaire de l'Ecole Normale Supérieure de Lyon, Unité Mixte de Recherche 5665, Centre National de la Recherche Scientifique/Ecole Normale Supérieure, Institut National de la Recherche Agronomique 913, 46 allée d'Italie, 69364 Lyon Cedex 07, France. E-mail: Irma.Machuca@ ens-lyon.fr.

This work was supported by the Center National de la Recherche Scientifique (Grant on Biologie Cellulaire: du normal au pathologique), the Association pour la Recherche Contre le Cancer, and the Ligue Contre le Cancer (Rhône et Nationale). Patrice Boissy was the recipient of a grant from the Ligue Contre le Cancer (Drôme) and from the Fondation pour la Recherche Médicale.

* Present address: Osteopro A/S, OsteoPark, Herlev Hovedgade 207, 2730 Herlev, Denmark.

References

1. **Aplin JD** 1991 Implantation, trophoblast differentiation and haemochorial placentation: mechanistic evidence in vivo and *in vitro*. J Cell Sci 99:681–692
2. **Vignery A** 2000 Osteoclasts and giant cells: macrophage-macrophage fusion mechanism. Int J Path 81:291–304
3. **Lee TT, Martin FC, Merrill JE** 1993 Lymphokine induction of rat microglia multinucleated giant cell formation. Glia 8:51–61
4. **Foe VE, Alberts BM** 1983 Studies of nuclear and cytoplasmic behaviour during the five mitotic cycles that precede gastrulation in *Drosophila* embryogenesis. J Cell Sci 61:31–70
5. **Berger F** 1999 Endosperm development. Curr Opin Plant Biol 2:28–32
6. **Mintz B, Baker WW** 1967 Normal mammalian muscle differentiation and gene control of isocitrate dehydrogenase synthesis. Proc Natl Acad Sci USA 58: 592–598
7. **Harris AJ, Duxson MJ, Fitzsimons RB, Rieger F** 1989 Myonuclear birthdates distinguish the origins of primary and secondary myotubes in embryonic mammalian skeletal muscles. Development 107:771–784
8. **Arai F, Miyamoto T, Ohneda O, Inada T, Sudo T, Brasel K, Miyata T, Anderson DM, Suda T** 1999 Commitment and differentiation of osteoclast precursor cells by the sequential expression of c-Fms and receptor activator of nuclear factor κB (RANK) receptors. J Exp Med 190:1741–1754
9. **Lacey DL, Timms E, Tan HL, Kelley MJ, Dunstan CR, Burgess T, Elliott R, Colombero A, Elliott G, Scully S, Hsu H, Sullivan J, Hawkins N, Davy E, Capparelli C, Eli A, Qian YX, Kaufman S, Sarosi I, Shalhoub V, Senaldi G, Guo J, Delaney J, Boyle WJ** 1998 Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. Cell 93:165–176
10. **Sexton PM, Findlay DM, Martin TJ** 1999 Calcitonin. Curr Med Chem 6:1067–1093
11. **Mundy** 1995 The morphology of osteoclast. In: Mundy GR, ed. Bone remodeling and its disorders. London: Martin Dunitz Ltd.; 12–13
12. **Roodman GD** 1996 Paget's disease and osteoclast biology. Bone 19:209–212
13. **Piper K, Boyde A, Jones SJ** 1992 The relationship between the number of nuclei of an osteoclast and its resorptive capability *in vitro*. Anat Embryol (Berl) 186:291–299
14. **Wansink DG, Schul W, van der Kraan I, van Steensel B, van Driel R, de Jong L** 1993 Fluorescent labeling of nascent RNA reveals transcription by RNA polymerase II in domains scattered throughout the nucleus. J Cell Biol 122: 283–293

15. **Bouniol C, Nguyen E, Debey P** 1995 Endogenous transcription occurs at the 1-cell stage in the mouse embryo. Exp Cell Res 218:57–62
16. **Solari F, Domenget C, Gire V, Woods C, Lazarides E, Rousset B, Jurdic P** 1995 Multinucleated cells can continuously generate mononucleated cells in the absence of mitosis: a study of cells of the avian osteoclast lineage. J Cell Sci 108:3233–3241
17. **Masson C, Bouniol C, Fomproix N, Szollosi MS, Debey P, Hernandez-Verdun D** 1996 Conditions favoring RNA polymerase I transcription in permeabilized cells. Exp Cell Res 226:114–125
18. **Bursztajn S, Berman SA, Gilbert W** 1989 Differential expression of acetylcholine receptor mRNA in nuclei of cultured muscle cells. Proc Natl Acad Sci USA 86:2928–2932
19. **Su X, Berman SA, Sullivan T, Bursztajn S** 1995 Myoblast and myotube nuclei display similar patterns of heterogeneous acetylcholine receptor subunit mRNA expression. J Cell Biochem 58:22–38
20. **Duclert A, Changeux JP** 1995 Acetylcholine receptor gene expression at the developing neuromuscular junction. Physiol Rev 75:339–368
21. **Solari F, Flamant F, Cherel Y, Wyers M, Jurdic P** 1996 The osteoclast generation: an *in vitro* and *in vivo* study with a genetically labelled avian monocytic cell line. J Cell Sci 109:1203–1213
22. **Kanehisa J, Heersche JN** 1988 Osteoclastic bone resorption: *in vitro* analysis of the rate of resorption and migration of individual osteoclasts. Bone 9:73–79
23. **Vaananen HK, Zhao H, Mulari M, Halleen JM** 2000 The cell biology of osteoclast function. J Cell Sci 113:377–381
24. **Wansink DG, Nelissen RL, de Jong L** 1994 *In vitro* splicing of pre-mRNA containing bromo-uridine. Mol Biol Reprod 19:109–113
25. **Baron R** 1999 Anatomy and ultrastructure of bone. In: Favus MJ, ed. Primer on metabolic bone diseases and disorders of mineral metabolism. Philadelphia: Lippincott-Raven Ltd.; 3–10
26. **Piper K, Boyde A, Jones SJ** 1995 Volumes of chick and rat osteoclasts cultured on glass. Calcif Tissue Int 56:382–389
27. **Fallon MD, Teitelbaum SL, Kahn AJ** 1983 Multinucleation enhances macrophage-mediated bone resorption. Lab Invest 49:159–164
28. **Zheng MH, Papadimitriou JM, Nicholson GC** 1991 RNA synthesis in isolated rat osteoclasts: inhibitory effect of calcitonin. Bone 12:317–322
29. **Chambers TJ, Magnus CJ** 1982 Calcitonin alters behaviour of isolated osteoclasts. J Pathol 136:27–39
30. **Kaji H, Sugimoto T, Miyauchi A, Fukase M, Tezuka K, Hakeda Y, Kumegawa M, Chihara K** 1994 Calcitonin inhibits osteopontin mRNA expression in isolated rabbit osteoclasts. Endocrinology 135:484–487