Estrogen Added Intermittently, but Not Continuously, Stimulates Differentiation and Bone Formation in SaOS-2 Cells

Leticia Gomez RAO,*[,a] Lily Jin-Fang LIU,[a] Timothy McCleod MURRAY,[a] Elizabeth MCDERMOTT,[b] and Xizhong ZHANG[a]

[a] *Calcium Research Laboratory, Division of Endocrinology and Metabolism, St. Michael's Hospital and Department of Medicine, University of Toronto; and* [b] *Department of Pathology, St. Michael's Hospital and Department of Medicine, University of Toronto; Toronto, Ontario Canada M5B 1A6.* Received September 20, 2002; accepted April 4, 2003

Although it is well established that estrogen inhibits bone resorption, its effects on bone formation remain controversial. We studied the effects of intermittent and continuous treatment with estrogen on bone formation *in vitro* using long term cultures of SaOS-2 cells under conditions that permit mineralization. SaOS-2 cells cultured in dexamethasone, ascorbic acid and β-glycerophosphate for up to 17 d formed mineralized bone nodules as visualized by von Kossa staining. Electron microscopic analysis of ultrathin sections of representative mineralized nodules showed the presence of mineral deposits, collagen fibrils and osteocytes. Both the mineralized nodule numbers and areas increased exponentially with time of culture after addition of β-glycerophophate at day 8. Intermittent addition of 17β-estradiol (E_2) for 6 h or 24 h of every 48 h starting at day 3 or day 8 to the end of culture period resulted in a specific time- and dose-dependent stimulation of mineralized bone nodule number and area, and alkaline phosphatase activity which were accompanied with increase in cell numbers. On the other hand, continuous treatment with E_2 added every 48 h had no effect. The estrogen receptor alpha (ERα) mRNA expression was stimulated after 6 or 24-h (intermittent), but not after 48-h (continuous) treatment with E_2. The stimulatory effect of E_2, when added intermittently, but not continuously, on differentiation and bone formation in human osteoblasts in culture may be relevant to previous reports of stimulatory effects of E_2 on bone formation *in vivo*.

Key words human osteoblast; estrogen; bone formation; SaOS-2 cells; mineralized bone nodule; differentiation

Estrogen deficiency has been established as the major cause of postmenopausal osteoporosis, a condition resulting from a disturbance in bone remodelling. Osteoporosis is characterized by low bone mass and deterioration of the microarchitecture of bone tissue with a consequent risk of fracture and debilitation.[1,2] Treatment with estrogen (E_2) can prevent the loss of bone among osteoporotic women,[3,4] and is the treatment of choice among postmenopausal women for the prevention of osteoporosis.[5] In spite of the accepted use of estrogen in the prevention of osteoporosis, the mechanisms involved in its action are still unclear. It is thought that the major effect of estrogen *in vivo* is inhibition of resorption,[6] but effects on bone formation have been reported in lower species.[7—11] More recently, studies[12,13] showing a sustained stimulation of osteoblast function in postmenopausal women who were exposed for prolonged period to relatively high doses of estrogen, may suggest that estrogen also has an effect on bone formation in humans.[6,14,15] Others still view the anabolic effect of estrogen as being controversial.[16] If indeed estrogen has anabolic effect *in vivo*, its mechanism of action requires elucidation.

Although a direct action of estrogen on cells of the osteoblast lineage has now been demonstrated the cellular and molecular mechanisms of its action are still not fully delineated.[17,18] Because of the notion that presence of low number of estrogen receptors in osteoblasts cause the negligible or often absence of responses to estrogen, manipulations have been carried out to increase the low abundance of estrogen receptor number in human osteoblasts, and the resultant cell models have been used to study the effects of estrogen.[19,20] The other way to increase estrogen receptor is by allowing the cells to differentiate in culture as we have done.[21—24] Thus, we have shown that the immunohistochemically detectable classical estrogen receptors (ERα), were detected in greater number in the more differentiated SaOS+Dex cells grown in the presence of dexamethasone compare with the less differentiatiated SaOS−Dex cells grown in its absence.[21] Furthermore, we have shown that the effect of estrogen on alkaline phosphatase activity, in combination with other hormones, is enhanced in the more differentiated cells,[22—24] confirming that high level of ERα increased the response of the cells to estrogen. By culturing SaOS+Dex cells in the presence of ascorbic acid, and β-glycerophosphate in order to obtain even more differentiated osteoblastic cells that have the property of mineralizing in culture, we were able to study the effects of hormones including parathyroid hormone (PTH)[25] and estrogen[26] as well as the effect of extracellular calcium[27] on mineralized bone nodule formation. Thus, as we have recently reviewed[17] and reported in a rapid communication,[28] we found that estrogen has a direct effect on bone formation in SaOS-2 cells when added intermittently. In this study, we report in more detail the action of intermittent treatment with E_2 and the comparison of this mode of addition with that of continuous addition on cell differentiation and bone formation in long term cultures of SaOS-2 cells.

MATERIALS AND METHODS

Cell Culture SaOS-2 cells (obtained from American Type Culture Collection, Rockville Md) were initially cultured in HAM's F-12 medium containing 10% fetal calf serum, HEPES buffer, pH 7.35, antibiotic and 1.4 mM $CaCl_2$ as we have previously described.[23] This is further supplemented with 10 nM dexamethasone (Dex) and 50 μg/ml ascorbic acid. At day 8, 10 mM β-glycerophosphate was added and the addi-

* To whom correspondence should be addressed. e-mail: leticia.rao@utoronto.ca

tion repeated at every medium change until the end of culture. When intermittent addition of E_2 was to be carried out, vehicle or varying concentrations of E_2 were added at day 3 or day 8 for the first 6 or 24 h, the medium then replaced fresh without the hormone, and then cultures continued for 48 h. The E_2 pulse was repeated every 48-h cycle until day 17. For continuous addition of E_2, varying concentrations of E_2 were added at each medium change from day 3 or day 8 until the end of the culture period.

Methylene Blue Method of Determining Cell Number A modified method of Genty *et al.*[29] was used. The cells fixed with 4% paraformaldehyde were rinsed with borate buffer, pH 8.5, stained for 10 min with 1% methylene blue prepared in 0.01 M borate buffer, pH 8.5, excess stain washed with the same buffer and the blue color stain of the nuclei were eluted with ethanol–0.01 M HCl (1 : 1). The optical density of the eluted color is measured in a Multiscan Spectrophotometer at 620 nm.

Determination of Mineralized Bone Nodule Formation The cells were fixed overnight with neutral buffered formalin and stained *in situ* using the standard von Kossa technique. The mineralized nodule areas and numbers were quantified using a LECO image analyzer (LECO, Toronto, Ontario, Canada).

Alkaline Phosphatase (ALP) Activity Assay The cells were washed twice with 50 mM Tris, pH 7.3, harvested by scraping, and ALP activity determined according to the method of Lowry[30] using cell sonicates as previously described.[23] The protein content of the cell sonicates was determined using a commercial protein dye reagent (Biorad).

Light Microscopy, Electron Microscopy and Electron Microprobe Analyses Von Kossa stained cells were examined and photographed using a Zeiss phase contrast microscope and camera. Cells cultured for 15 d in cyclopore membrane (VWR) wells were prefixed overnight in 2.5% glutaraldehyde in Sorensen's phosphate buffer, pH 7.4. The membrane was excised and the fixed cells postfixed with 1% osmium tetroxide, dehydrated in graded ethanol and flat embedded in an Epon–Araldite mixture. 70—90 nm ultrathin sections were cut on a Sorvall MT2-B ultramicrotome, stained with uranyl acetate and lead citrate, and examined with a Philips 410LS transmission electron microscope. Electron diffraction was performed on the same section on a Philips EM430 Transmission Electron Microscope.

RNA Extraction and RT-PCR Total RNA was extracted from cultures of SaOS cells treated with vehicle or 10 nM E_2 using the method of Chomczynski and Sacchi.[31] cDNA was synthesized by reverse transcription (RT) from 1—3 μg of total RNA in a 20 μl reaction mixture containing 1x reverse transcriptase buffer [10x = 100 mM Tris–HCl, pH 8.8; 500 mM KCl and 1% Triton X-100], 5 mM $MgCl_2$, dCTP, dGTP, dATP and dTTP, each at 1 mM; 20 U of RNAse inhibitor, 15 U of AMV reverse transcriptase and 0.5 μg of Oligo (dT) Primer (Promega, Madison, WI, U.S.A.). Reaction times were 1 h at 42 °C. PCRs were then performed on each RT reaction, using ERα specific oligonucleotide primers (0.4 μM) in 50 μl containing 1.5 mM $MgCl_2$, 1x Taq DNA Polymerase Buffer [10x = 20 mM Tris–HCl, pH 8.0; 100 mM KCl; 0.1 mM EDTA; 1 mM DTT; 50% glycerol; 0.5% Tween 20 and 0.5% Nonidet P40], dCTP, dGTP, dATP and dTTP, each at 200 μM 1.25 U of Taq polymerase (Promega, Madison, WI, U.S.A.) and GAPDH 5′ and 3′ primer as internal quantitative control. Each cDNA sample was run in triplicate for every PCR. Amplification was carried out for sixty cycles (30 s 94 °C, 20 s 55 °C, 45 s 72 °C). All PCR products were electrophoresed on a 1.5% agarose gel containing 0.02 μg/ml ethidium bromide and photographed under UV light using a Polaroid ISO 3000/36° film. The intensities and total area of the bands present on the photograph were analysed on LECO image analysing system. The quantitative differences between cDNA samples were calculated after normalising in the corresponding GAPDH PCR product. The primers for ERα[32] and ERβ[33] were chosen in the N-terminal A/B region, spanning one intron from published sequences as follows:

(1) ERα, (345 bp): sense; 5′-AATTCAGATAATCGACGCCAG
antisense; 5′-GTGTTTCAACATTCTCCCTCCTC.

(2) ERβ, (259 bp): sense; 5′-TTCCCAGCAATGTCACTAACT
antisense; 5′-CTCTTTGAACCTGGACCAGTA.

(3) GAPDH, (608 bp): sense; 5′-GTCAACGGATTTGGTCGTAT
antisense: 5′-GAGGCAGGGATGATGTTCTG.

RESULTS

Characteristics of the Mineralized Bone Nodules in 17-d Cultures of SaOS-2 Cells The mineralized bone nodule formation in SaOS-2 cells cultured for 17 d in medium supplemented with Dex, ascorbic acid and β-glycerophosphate is illustrated in Figs. 1 to 3. At day 17, the mineralized nodules could be visualized with the naked eye as discrete black nodules as a result of von Kossa staining illustrated in the photograph shown in Fig. 1A. The discrete nodules were generally distributed throughout the culture dish. An examination under phase contrast light microscope of the cultures at various periods of time showed that the cells started multilayering at day 6 (data not shown). The von Kossa-stained mineralized nodules could be quantitated as early as day 10, or two days after the addition of β-glycerophosphate as will be discussed below. A phase contrast photomicrograph of representative mineralized nodules at day 17 is shown in Fig. 1B. The nodules were three dimensional in appearance and discrete, making them amenable to quantitation by image analysis. Representative electron photomicrographs of ultra thin sections of some nodules are depicted in Figs. 2A to C. Figure 2A demonstrates mineral deposits and collagen fibrils in the extracellular spaces. Figure 2B is a high magnification of an area which demonstrate collagen banding. In Fig. 2C an osteocyte process embedded in the collagen matrix and mineral deposits can be visualized. The mineral deposits have been demonstrated to have the characteristic crystalline structure of hydroxyapatite as shown by the electron diffraction pattern in Fig 3.

Effects of Intermittent Exposure to E_2 To determine the effect of intermittent addition of E_2 on cell proliferation and differentiation of SaOS-2 cells, the cell number and the development of ALP activity were followed over time in cul-

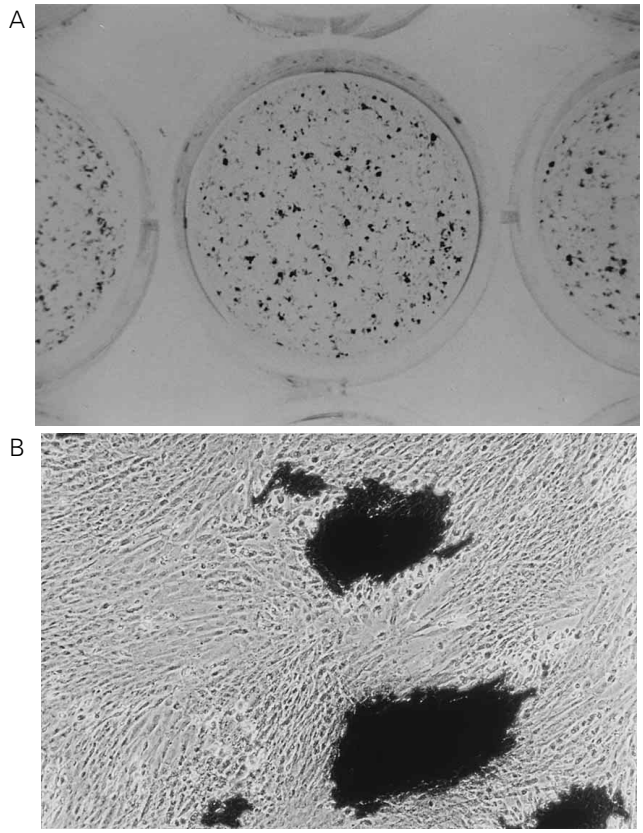

Fig. 1. (A) Photograph of a Fixed Culture of SaOS-2 Cells Stained with the von Kossa Technique. Each black stain represents discrete mineralized bone nodule
(B) Phase Contrast Photomicrograph of Representative Mineralized Bone Nodules (Magnification, ×40).

ture, in the absence or presence of E_2. Intermittent addition was carried out by adding E_2 at day 3 for 6 h of every 48 h of culture as described above. With this mode of treatment with vehicle, the cell number increased exponentially over time from day 3 to day 10 of cultures, then decrease from day 13 to day 17. The cell number was stimulated by intermittent addition of E_2 from day 13 to day 17 (Fig. 4). ALP activity was followed over time in the absence or presence of intermittently added E_2 (Fig. 5A). ALP activity increased with time of culture by over the culture period from day 3 to day 13. ALP activity was maximal at day 13, and remained constant until day 17 (Fig. 5A). Intermittent addition of 10 nM E_2 showed an ALP activity increase of 9-fold compared to the 7.2-fold increase in the vehicle alone, over the time periods tested from day 3 to day 17 [Fig. 5A, Two-way analysis of variance (ANOVA), For E_2 effect: $n=6$, $F=29.26$, $p<0.0001$ and for time course effect: $n=6$, $F=190.6$, $p<0.001$]. We then tested the effects of E_2 on ALP activity at concentrations from 0.1 nM to 1 μM. Figure 5B showed that intermittent addition of E_2 dose-dependently stimulated ALP activity in SaOS-2 [Fig. 5B, One-way ANOVA, $n=6$, $F=8.95$, $p<0.01$], with significance at 10 nM and 1 μM of E_2, both at $p<0.05$.

The formation of mineralized bone nodules and the effects thereon of intermittently added E_2 were followed over time in cultures of SaOS-2 cells (Fig. 6). At each time point, the cells were fixed, stained with von Kossa and the nodule number (Fig. 6A) and area (Fig. 6B) analyzed and quantified

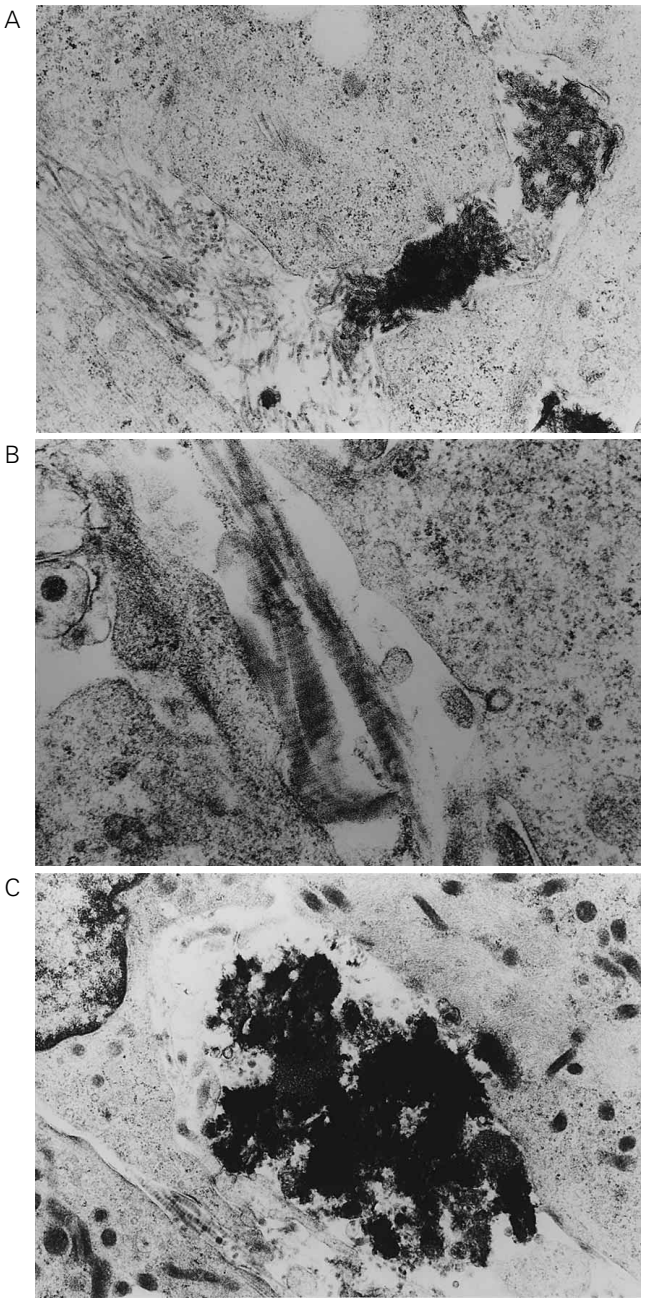

Fig. 2. Electron Photomicrographs of Ultrathin Sections of Representative Bone Nodules Illustrating (A) Mineral Deposits and Banded Collagen Fibrils in the Extracellular Spaces (Magnification, 21000×), (B) an Area Which Demonstrates Collagen Banding (Magnification, 84000×) and (C) Osteocyte Process Embedded in the Collagen Matrix and Mineral Deposits (Magnification, 21000×)

by image analyser. Very few von Kossa-stained mineralized nodules could be visualized at day 10, which was 2 d after the addition of β-glycerophosphate, and the earliest time point examined. The nodule number (Fig. 6A) and nodule area (Fig. 6B) increased exponentially with time of culture up to 17 d, the last time point studied. From day 10 to day 17 of cultures when the cultures received vehicle alone, there was a 22.4-fold increase in nodule numbers (Fig. 6A) compared to 34.4-fold increase in cultures that received intermittent E_2 (Fig. 6A). In terms of mineralized nodule areas, these increases are 12.9-fold for vehicle alone and 22.2-fold for in-

Fig. 3. An Electron Diffraction Pattern of a Mineral Deposit Demonstrating the Characteristic Crystalline Structure of Hydroxyapatite

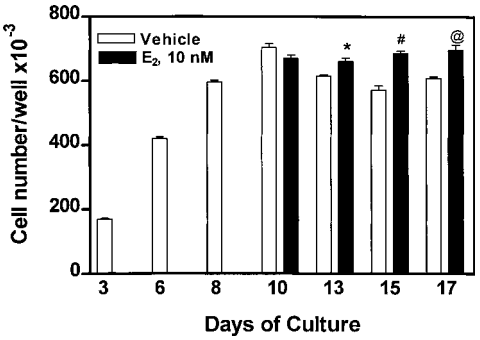

Fig. 4. Stimulatory Effect of Intermittent Addition of E_2 on the Cell Numbers of SaOS-2 Cells

Vehicle or 10 nM E_2 was pulsed intermittently for 6 h from day 8 and every 48 h thereafter as described under Methods. At each time point, the cells were fixed and the cell number determined by the methylene blue method as described in the text. Each point is a mean±S.E.M. of 4 determinations. *, $p<0.05$; #, $p<0.001$, @, $p<0.01$.

termittently added E_2 (Fig. 6B). Thus, intermittent addition of E_2 stimulated bone nodule numbers [Fig. 6A, two way ANOVA, For E_2 effect: $n=6$, $F=101.7$, $p<0.0001$ and for time-course effect: $n=6$, $F=240.8$, $p<0.001$] as well as nodule area [Fig. 6B, two way ANOVA, For E_2 effect: $n=6$, $F=53.93$, $p<0.0001$ and for time course effect: $n=6$, $F=117.5$, $P<0.0001$] when compared with vehicle alone, in a time-dependent manner from days 10 to 17.

The experiments were next carried out with varying doses of E_2. The intermittent treatment with E_2 dose-dependently stimulated the mineralized bone nodule formation in SaOS-2 cells (Fig. 7), both in terms of mineralized nodule numbers [Fig. 7A, one-way ANOVA, $n=6$, $F=6.88$, $p<0.005$] and nodule area [Fig. 7B, $n=6$, $F=5.73$, $p<0.01$], with significant stimulation for both nodule number and area at 10 nM and 1 μM E_2 (both, significant at $p<0.05$).

We then tested whether the time period at which the intermittent addition of E_2 was started would make a difference in the responses observed. To study this, E_2 addition was started either at day 3 or day 8 of culture, and the mineralized nodule formation and ALP activity determined at day 17. Table 1 shows that E_2 added intermittently, starting at day 3 or day 8, both resulted in dose-dependent stimulatory effect on nodule number: and area and ALP activity [Day 3: nodule number, $n=6$, $F=7.24$, $p<0.005$; nodule area, $n=6$, $F=9.16$, $p<0.001$ and ALP activity, $n=6$, $F=8.83$, $p<0.005$] and [Day 8: nodule number, $n=6$, $F=6.88$, $p<0.005$; nodule area, $n=6$,

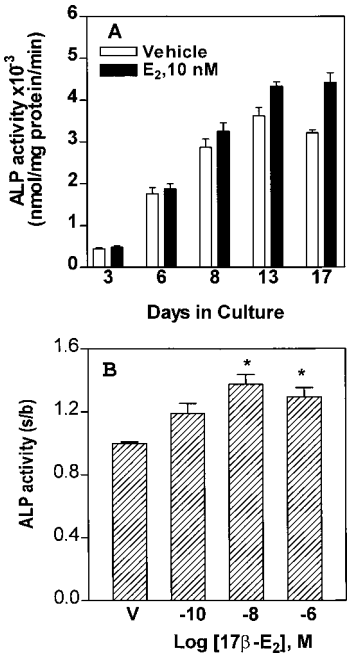

Fig. 5. Time Course (A) and Dose-Dependent (B) Stimulatory Effects of Intermittent Addition of E_2 on ALP in SaOS-2 Cells Treated as Described in the Text. *$p<0.01$

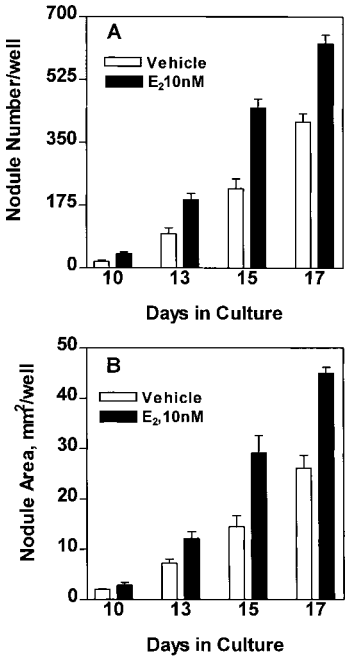

Fig. 6. Time Course of the Stimulatory Effect of Intermittent Addition of E_2 on Mineralized Bone Nodule (A) Number and (B) Area

Vehicle or 10 nM E_2 was pulsed intermittently for 6 h in a similar manner as in Fig. 4. At each time point, the cells were fixed, stained with Von Kossa and the mineralized nodules quantified by image analyzer. Each point is a mean±S.E.M. of determinations.

$F=5.73$, $p<0.01$; and ALP activity, $n=6$, $F=8.95$, $p<0.005$].

Although the results presented were from experiments carried out in which E_2 was added intermittently for 6 h every 48-h medium change, we can see from Table 2 that a stimulatory effect was also observed when E_2 was added intermittently every 24 h, instead of every 6 h.

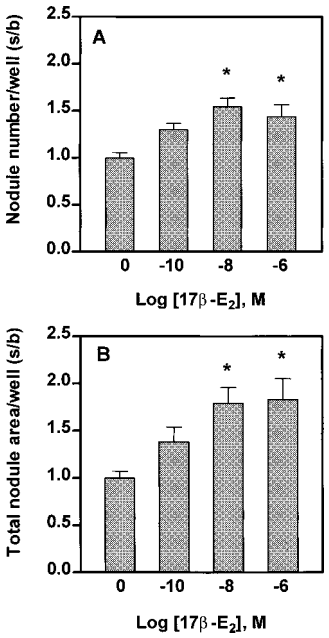

Fig. 7. Dose-Dependent Stimulatory Effects of Intermittent Addition of E_2 on Mineralized Bone Nodule (A) Number and (B) Area

SaOS-2 cells were treated with intermittent addition of increasing concentrations of E_2 as described in the text and the incubations stopped and analysed at day 17. *, $p<0.05$.

Effects of Continuous Exposure to E_2 To test the effect of continuous addition of E_2 on cell proliferation and differentiation of SaOS-2 cells, E_2 was added to the cultures starting from day 3 and, unlike the intermittent treatment, E_2 was present in the medium throughout the 48 h culture period, with re-addition of fresh E_2 at every 48-h medium change thereafter. The cell number increased exponentially over time from day 3 to day 10 of cultures, then decreased and levelled off thereafter from day 13 to day 17 (Fig. 8). The E_2-treated cells had the same number of cells as the untreated cells at day 10 to day 17, indicating that continuous presence of E_2 had no effect on the proliferation of SaOS-2 cells (Fig. 8), unlike the effect observed when E_2 was added intermittently (Fig. 4). In contrast to the results obtained with the experiments in which E_2 was added intermittently, continuous addition of E_2, from day 3 to the end of the culture period did not have any significant time- (Fig. 9A) or dose-dependent (Fig. 9B) effects on ALP activity. Similarly, there was no significance in the observed time course (Figs. 10A, B) and dose-dependent (Figs. 11A, B) effects of E_2 on nodule numbers (Figs. 10A, 11A, respectively) or nodule area (Figs. 10B, 11B, respectively).

Specificity of 17β-Estradiol The effect of 17β-estradiol was compared to that of the inactive analog 17α-estradiol, both after intermittent and continuous addition to SaOS-2 cells starting from day 3 of culture. Table 3 shows that whereas intermittent addition of 17β-estradiol had significant stimulatory effects over vehicle treatment on mineralized

Table 1. Effect of E_2 Added Intermittently to SaOS-2 Cells at Different Days in Culture on Mineralized Bone Nodule Formation and ALP Activity[a)]

Day of culture at which vehicle or E_2 was added	Log [E_2], M	Mineralized nodules (s/b)		ALP activity (s/b)
		Number	Total area	
Day 3	Vehicle	1.00±0.06	1.00±0.07	1.00±0.01
	−10	1.21±0.06	1.21±0.11	1.19±0.06
	−8	1.54±0.09	1.79±0.16	1.37±0.06
	−6	1.43±0.13	1.72±0.15	1.29±0.06
Day 8	Vehicle	1.00±0.55	1.00±0.07	1.00±0.01
	−10	1.30±0.63	1.38±0.16	1.19±0.07
	−8	1.54±0.09	1.79±0.17	1.37±0.06
	−6	1.43±0.13	1.83±0.22	1.29±0.06

a) SaOS-2 cells were cultured as described under Methods and vehicle or varying concentrations of E_2 were added at day 3 or day 8 for the first 6 h, the medium then replaced fresh without the hormone and then cultures continued for 48 h. The addition was repeated every 48 h cycle until day 17 and the indicated parameters assayed as described under Methods. Both additions at day 3 or day 8 resulted in dose-dependent stimulatory effects on nodule number and area and ALP activity. The statistical significance is discussed under Results section. Each point represents mean E_2 treated/vehicle (s/b)±S.E.M., $n=6$ from representative of 2 experiments, each with 3 determinations.

Table 2. Effect of E_2 Added Intermittently at Day 8 for Different Periods of Time to SaOS-2 Cells on Mineralized Bone Nodule Formation and ALP Activity[a)]

Mode of treatment	Vehicle or estrogen	Mineralized nodules (s/b)		ALP activity (s/b)
		Number	Total area	
6-h intermittent ($n=6$)	Vehicle	1.000±0.049	1.000±0.052	1.000±0.028
	17β-E_2	1.425±0.114[b)]	1.501±0.092[d)]	1.116±0.047[f)]
24-h intermittent ($n=12$)	Vehicle	1.000±0.035	1.000±0.003	1.000±0.038
	17β-E_2	1.231±0.055[c)]	1.556±0.093[e)]	1.250±0.063[c)]

a) SaOS-2 cells were cultured as described under Methods, then vehicle or 10 nM 17β-E_2 was added intermittently for 6 or 24 h at day 8, the cultures terminated at day 17 and the indicated parameters assayed as described under Methods. Each point represents mean treated/vehicle (T/V)±S.E.M. from 2 to 4 experiments of 3 determinations each. Statistical significance using *t*-test analysis: treatment *vs.* vehicle: *b*) $p<0.005$; *c*) $p<0.01$; *d*) $p<0.0001$; *e*) $p<0.0005$; *f*) $p<0.05$.

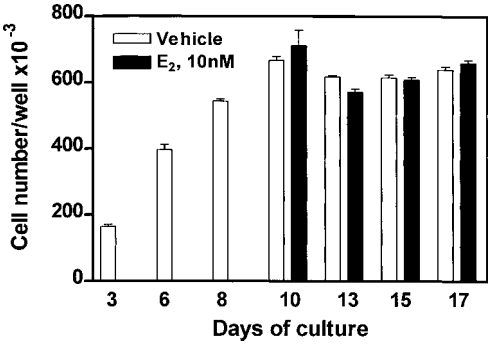

Fig. 8. Absence of a Time Course Effect of Continuous Addition of E_2 on the Cell Numbers of SaOS-2 Cells

Vehicle or 10 nM E_2 was added continuously from day 8 and every 48 h thereafter as described under Methods. At each time point, the cells were fixed and the cell numbers assayed by the methylene blue method as described in the text under Materials and Methods.

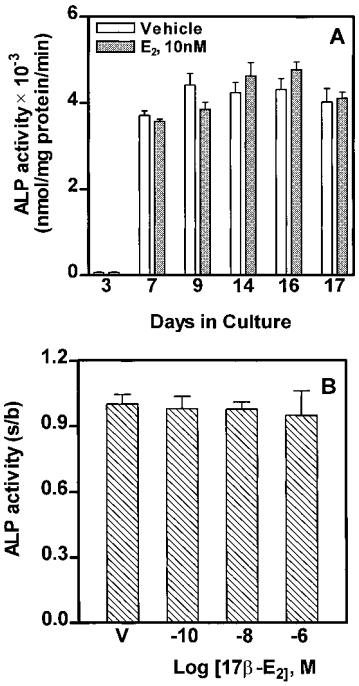

Fig. 9. Absence of Time Course (A) and Dose-Dependent (B) Effects of Continuous Addition of E_2 on ALP Activity in SaOS-2 Cells Treated as Described under Fig. 7

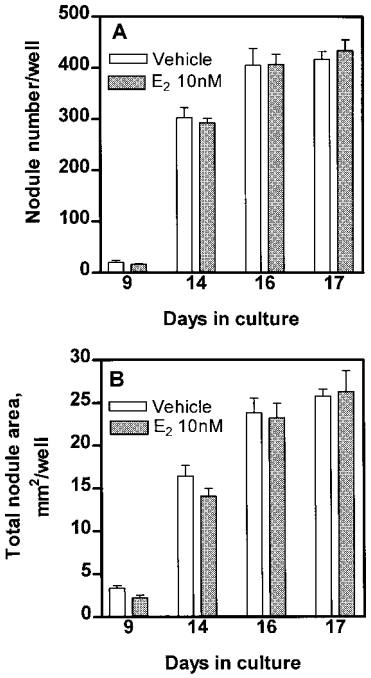

Fig. 10. Absence of a Time Course Effect of Continuous Addition of E_2 on Mineralized Bone Nodule (A) Number and (B) Area

Vehicle or 10 nM E_2 was added at day 3 and at every 48 h of medium change as described under Methods. At each time point, the cells were fixed, stained with Von Kossa and the mineralized nodules quantified by image analyzer.

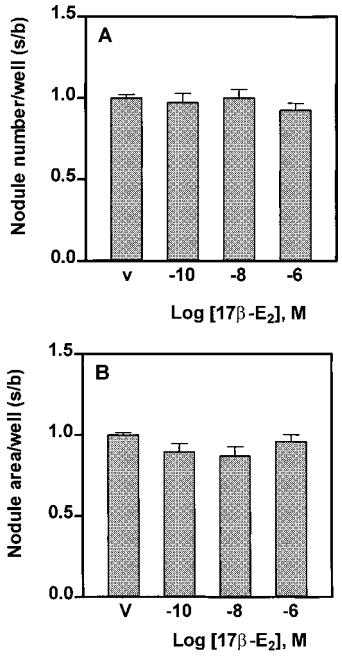

Fig. 11. Absence of a Dose-Dependent Effects of Continuous Addition of E_2 on Mineralized Bone Nodule (A) Number and (B) Area

Continuous addition of E_2 was carried out as described in Fig. 7.

bone nodule numbers ($p<0.005$) and areas ($p<0.0005$) and ALP activity ($p<0.05$), similar treatment with 17α-estradiol produced no significant effects on these paramenters. Continuous addition of either 17β-estradiol or 17α-estradiol did not result in any significant effects on nodule number and area and ALP activity up to a concentration of 1 μM, (Table 2).

Effects of E_2 on ERα mRNA Expression The effects of E_2 on ERα mRNA expression were observed when it was added at day 8 or day 16. To mimic intermittent addition of E_2, the RNA was extracted after 24 h and to mimic continuous addition, the RNA was extracted after 48 h. The results of RT-PCR amplification using the primers for ERα are shown in Figs. 12A and B. The figures revealed that 24-h treatment with E_2 mimicking intermittent addition, resulted in a statistically significant stimulation of ERα mRNA Expression both when added at day 8 (Fig. 12A) or day 16 (Fig. 12B). On the other hand, after 48 h of treatment with E_2, mimicking a continuous addition, ERα mRNA Expression was not different from the vehicle treatment. ERβ of the same basepair length as that published in the literature[32] could not be detected.

Table 3. Specificity of Estrogen Effect: Effect of 17β-E_2 or 17α-E_2 on Mineralized Bone Nodule Formation and ALP Activity[a)]

Mode of treatment	Vehicle or estrogen	Mineralized nodules (s/b)		ALP activity (s/b)
		Number	Total area	
6-h intermittent exposure	Vehicle	1.00±0.07	1.00±0.08	1.00±0.04
	17α-E_2	0.93±0.08	1.02±0.07	1.02±0.02
	Vehicle	1.00±0.05	1.00±0.05	1.00±0.03
	17β-E_2	1.43±0.11*	1.50±0.09**	1.12±0.05***
Continuous exposure	Vehicle	1.00±0.17	1.00±0.09	1.00±0.02
	17α-E_2	1.13±0.19	0.89±0.18	0.99±0.03
	Vehicle	1.00±0.06	1.00±0.06	1.00±0.04
	17β-E_2	0.81±0.10	0.77±0.11	0.96±0.03

a) SaOS-2 cells were cultured as described under Methods, then vehicle, 10 nM 17β-E_2 or 17α-E_2 was added at day 3 either intermittently or continuously, the cultures terminated at day 17 and the indicated parameters assayed as described under Methods. Each point represents mean E_2 treated/vehicle (s/b)±S.E.M., n=6, from representative of 2 experiments each with 3 determinations. Statistical significance using t-test analysis: treatment *vs.* vehicle: *$p<0.05$; **$p<0.0005$; ***$p<0.05$.

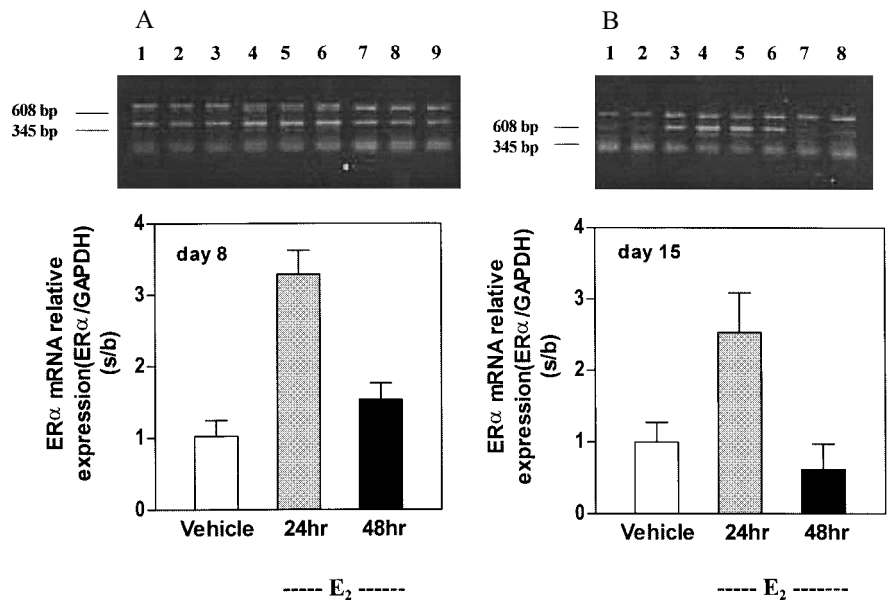

Fig. 12. Effects of E_2 on ERα mRNA Expression

Vehicle or 10 nM E_2 was added at day 8 (A) or day 15 (B), RNA extracted after 24 h and 48 h, RT-PCR performed, the product separated on agarose gel and the bands quatified by image analysis as described in the text. A (lanes 1—3) and B (lanes 1—3), vehicle; A (lanes 4—6) and B (lanes 4—6), 24 h with 10 nM E_2, A (lanes 7—9) and B (lanes 7—8), 48 h with 10 nM E_2. t-test: E_2 treatment for 24 h significantly higher than vehicle, $p<0.0005$; E_2 treatment for 48 h significantly greater than vehicle, $p<0.05$.

DISCUSSION

In this study we report that intermittent, but not continuous, exposure to E_2 resulted in a stimulation of differentiation and mineralized bone nodule formation in long term cultures of SaOS-2 cells. This stimulatory effect of E_2 provides strong *in vitro* evidence that a direct effect of E_2 on human osteoblasts *via* ERα maybe one of the mechanisms involved in its stimulatory effect on bone formation *in vivo*, seen both in lower species[7—11)] and in postmenopausal women.[12—15)] Furthermore, our data demonstrating the formation of mineralized bone-like structures in long-term cultures of SaOS-2 cells in the presence of dexamethasone, ascorbic acid and β-glycerophosphate make the SaOS-2 cell culture a valid human cell system for quantitative study of estrogen action on bone formation *in vitro*.

A number of cell systems using osteoblasts from lower species[34—36)] and human species[28,36—38)] are available for studying bone formation *in vitro*. The deposition of mineralized matrix[39)] and the spontaneous release of matrix-vesicle-like structures with the capacity to mineralize[40)] in long term cultures of SaOS-2 cells have also been reported previously. However, our results differ from the last two studies in that our long-term cultures of SaOS-2 cells formed discrete mineralized bone nodules capable of being quantified. Furthermore, the regulation by estrogen of these mineralized nodule formation in SaOS-2 mineralizing cultures was not studied.

In earlier work we reported the establishment of cell models of two different stages of differentiation by culturing them in the absence (SaOS−Dex cells) and presence (SaOS+Dex cells) of dexamethasone.[21—24)] We later demonstrated that the more differentiated SaOS+Dex cells had greater immunohistochemically detectable ERα.[21)] In this study we have further established and characterized a third and more differentiated model of cells that mineralizes in culture, by culturing SaOS+Dex cells in the presence of ascorbic acid and β-glycerophosphate. SaOS−Dex cells cultured in this manner did not mineralize in culture (data not shown). Although we did not carry

out any immunohistochemical localization of ERα in these cells, we were able to show using RT-PCR technique that the mineralizing SaOS+Dex cells express ERα. On the other hand, we have shown previously that E_2 had no effect on ER (presumably ERα) in the less differentiation SaOS−Dex.[21] The stimulatory effect of intermittently added E_2 on mineralized bone nodule formation in these more differentiated cells provide stronger evidence for our previous hypothesis[21—24] that the effect of E_2 is differentiation-stage dependent.

The mechanisms involved in the differential response of the cells to continuous *vs.* intermittent treatment with E_2 are not clear at the present time. However, we can speculate on several possibilities. Such differential effects could be a result of the differences in the effects on proliferation/differentiation of the osteoprogenitor cells, ERα synthesis, apoptosis of osteoblasts, growth factor synthesis, Cbfa1 synthesis or signal transduction signalling as discussed below in detail. One or a combination of these effects could be a possibility.

The stimulatory effect of intermittent addition of E_2 on mineralize bone nodule formation could be a result of its effect on the proliferation and/or differentiation of osteoprogenitor cells as demonstrated by the parallel effect on ALP activity, a marker of osteoblastic differentiation. Bellows and Aubin[41] showed that each nodule formed in rat calvarial osteoblast culture is derived from a single osteoprogenitor cell that proliferated and differentiated into mature bone forming osteoblasts. Although SaOS-2 cells are a clonal cell line, we[21—24] and others[42,43] have shown that SaOS-2 cells have the potential and capacity to differentiate into various stages in culture. Another clonal cell line that was shown to differentiate and mineralize in culture was the mouse MC3T3-E1 cells.[34] Since E_2 was added at day 3 or day 8 when the osteoprogenitor number is still increasing, we can speculate that the effect of E_2 is to promote a further increase in the pool of osteoprogenitors and/or further differentiation of the cells into bone-forming mature osteoblasts. The stimulatory effect of intermittently added E_2 on cell number may indicate that its effect is to increase the pool of osteoprogenitor cells. However, the effect of intermittent treatment with E_2 on further differentiation of the cells towards bone forming mature osteoblasts can not be ruled out. E_2 added in a continuous fashion did not have an effect on osteoprogenitor cells or further differentiation of the progenitor cells as evident from lack of effect by this mode of treatment on cell number, ALP activity and mineralized bone nodule formation.

Another mechanism by which E_2 exerts its effect on osteoblast number is *via* apoptosis or cell death. It has been reported that E_2 stimulated ERα mRNA and protein expressions and inhibited apoptosis in mesenchymal stem cell (MSC) cultures from ovariectomized (OVXed) and sham-operated mice.[44] Cooper *et al.*[45] suggested that the reduction of staurosporin-induced apoptosis in the murine ER-transformed SMER14 osteoblastic cell line with E_2 pretreatment could be a possible mechanism for increasing and/or maintaining the number of viable osteoblasts in bone. Indeed, much research is being carried out to delineate the role of apoptosis in the pathogenesis of osteoporosis.[46,47] Thus, a differential inhibition of apoptosis of SaOS-2 cells with intermittent, but not continuous, treatment with E_2 is a likely explanation for the difference in the effect of intermittent and continuous treatment with E_2 on cell number in SaOS-2 cultures.

Differences on the effect of mineralized bone nodule formation were also observed between continuous and intermittent treatment of osteoblasts with PTH.[48] It was shown in that study that the PTH effect may be mediated either by the signalling pathway protein kinase C (PKC) or protein kinase A (PKA), depending on the mode of addition.[47] Such possibilities in the case of E_2 action need to be explored since it has been reported that there is an interaction between the PKC and ER signalling systems in bone cells and that this interaction may be influenced by the proliferative and/or differentiative state of the cells, resulting in modulation of hormone responsiveness.[49]

We have shown in earlier studies that culturing SaOS-2 cells in the presence of Dex caused an increase in immunohistochemically localized ERα.[21] These data may imply that the effect of E_2 on the bone formation we observed in this study is mediated *via* ERα. In this study, we found that E_2 stimulated the message expression of ERα after 24 h of treatment, but this expression was decreased after 48 h, whether E_2 was added at day 8 or at day 16 of culture. The increase of ERα mRNA expression seen after 24 h is consistent with the stimulatory effect seen when E_2 was added intermittently for 24 h, whereas the decrease in the ER mRNA expression after 48 h of E_2 treatment may explain the absence of response to continuous mode of addition when E_2 was present in the medium throughout the 48 h period. Other laboratory[36] reported that the presence of 10 nM E_2 for 4 d resulted in an inhibition of mineralized matrix formation in hFOB/ERα9 cells, a human osteoblast cell line stably transfected with ERα. However, in that study, it was not determined whether the inhibitory effect by E_2 was accompanied by inhibition of the activity and/or further synthesis of ERα in these cells, and therefore it is uncertain if this inhibitory effect is mediated *via* ERα. We have earlier reported a similar inhibition in SaOS-2 cells treated continuously with E_2, but the inhibition[25] was small and not consistently found as we report in this study. The participation of the ERβ may also be possible in view of the report that the expression of ERβ mRNA increased during differentiation of human osteoblasts SV-HFO cells, and particularly at the mineralization stage.[50] On the other hand, Zhou *et al.*[44] showed that E_2 treatment of MSC cells from OVXed and sham-operated mouse resulted in a decrease in ERβ mRNA and protein expression which was accompanied by upregulation of osteogenic genes. In our present study we were unable to detect the ERβ mRNA of the same basepair length reported in the literature,[33] either because they are expressed at a low level to be detectable or are not present in the SaOS-2 cell cultures. Therefore, the involvement of ERβ in the E_2 action in our system can be excluded. This conclusion is compatible with the finding by Waters *et al.*[38] that E_2 had no regulatory effect on the matrix mineralization of the hOB/ERβ6 cells, cells that have been stably transfected with ERβ

It is now well established that the actions of E_2, both *in vivo* and *in vitro*, on osteoblasts of lower species[51,52] and human origins[17] are modulated by a number of growth factors including IGF-1, TGF-β BMP-6[53] and PDGF.[14] The effects of E_2 on the growth factor mRNA and protein expressions appear to be observed in more mature or matrix-producing osteoblasts[54,55] or osteoblast cell lines expressing

high levels of ERα as a result of transfection.[56] The growth factors, are in turn believed to act as coupling factors in a paracrine and autocrine fashion during bone resorption[57] and bone formation.[58] The mechanisms by which E_2 stimulates their message and protein expressions and how they in turn affect bone formation are not fully understood. However, for some of these growth factors, the stimulatory effect of E_2 may be related to the cross-talk in the signalling pathways between that for E_2/ERα and the growth factors/growth factor receptors and/or growth factor binding proteins[56,59] or its interaction with other hormones.[17,60] Although we did not study the regulation of growth factor expression by E_2 in our cultures, there is distinct possibility that intermittent and continuous addition of E_2 differ in their effects on the synthesis of growth factors, as well, as the signalling pathways involved.

Another possible explanation for the observed difference in the effects of intermittent and continuous treatment with E_2 could be the difference in their effects on Cbfa1. Cbfa1, a member of the osf2/runt/Cbfa family of transcription factor, has been shown to be critical for bone formation because of it's role in osteoblast recruitment and differentiation.[61] Cbfa1 has now been shown to regulate the expression of all the major genes expressed by osteoblasts[62] by binding to the binding site known as OSE2 present in these genes.[63] It has been reported that estrogen-induced osteogenesis is associated with the appearance of a population of Cbfa1-expressing cells within bone marrow[11] and in MSCs cultured from OVXed and sham operated mice.[44] Therefore, there is a possibility that intermittent, but not continuous, treatment with E_2 can stimulate the expression of Cbfa1 which could in turn stimulate the expression of growth factors, alkaline phosphatase, collagen type I and osteocalcin during the differentiation and mineralized bone nodule formation in SaOS-2 cells. However, this is presently a speculation and should be tested.

There is strong evidence to show that prevention of bone loss among osteoporotic patients by the conventional treatment with E_2 is due for the major part to its ability to inhibit bone resorption.[3,4] Tobias and Compston,[6] however, noted that long term high dose treatment of postmenopausal women with E_2 stimulated bone formation which was shown to be a result of its effect on osteoblast function.[14] The high dose E_2 was administered to osteoporotic women through transdermal implant which lasted anywhere from 12 to 15 years. Presumbly, this mode would be considered continuous treatment with estrogen. In our study, albeit *in vitro*, we showed that continuous treatment with E_2 did not have any effect on bone formation. However, the length of continuous treatment that SaOS-2 cells were subjected to may not be long enough to observe the anabolic effect of E_2. Our hypothesis, based on our present finding, is that it might be possible that shorter intermittent treatment with low dose estrogen to postmenopausal women, *e.g.* once a week oral dose, may produce the desired anabolic effect in a shorter period of time.

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