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Progressive changes in the protein composition of the nuclear matrix during rat osteoblast differentiation

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ABSTRACT **Primary cultures of fetal rat calvarial osteoblasts undergo a developmental sequence with respect to the temporal expression of genes encoding osteoblast phenotypic markers. Based on previous suggestions that gene–nuclear matrix associations are involved in regulating cell- and tissue-specific gene expression, we investigated the protein composition of the nuclear matrix during this developmental sequence by using high-resolution two-dimensional gel electrophoresis. The nuclear matrix was isolated at times during a 4-week culture period that represent the three principal osteoblast phenotypic stages: proliferation, extracellular matrix (ECM) maturation, and mineralization. The most dramatic changes in the nuclear matrix protein patterns occurred during transitions from the proliferation to the ECM maturation stage and from ECM maturation to the mineralization period, with only minor variations in the profiles within each period. These stage-specific changes, corresponding to the major transition points in gene expression, indicate that the nuclear matrix proteins reflect the progressive differentiation of the bone cell phenotype. Subcultivation of primary cells delays mineralization, and a corresponding delay was observed for the nuclear matrix protein patterns. Thus, the sequential changes in protein composition of the nuclear matrix that occur during osteoblast differentiation represent distinct stage-specific markers for maturation of the osteoblast to an osteocytic cell in a bone-like mineralized ECM. These changes are consistent with a functional involvement of the nuclear matrix in mediating modifications of developmental gene expression.**

The nuclear matrix is clearly visualized in resinless electron microscopy sections and whole mounts. After removal of soluble and cytoskeleton proteins and chromatin by sequential extraction, the matrix appears as a network of thick, polymorphic anastomosing filaments (1, 2). Further extraction of the nuclear matrix removes most matrix proteins and reveals a network of 9- and 13-nm core filaments, which contain most nuclear RNA as a structural component (3). A possible role for the nuclear matrix in tissue- and cell-type-specific gene expression is suggested by the recent observations by Fey and Penman (4) and Stuurman *et al.* (5) that the nuclear matrix protein composition is distinct in different tissues and cell types. Other indications of a functional involvement of the nuclear matrix in the regulation of gene expression include the following: preferential association with actively transcribed genes (6, 7), association of steroid receptors with the nuclear matrix (8, 9), association with heterogeneous nuclear RNA (10), RNA synthesis, and pre-mRNA splicing (11).

The culture of enzymatically isolated fetal rat calvarial osteoblasts that produces a mineralized bone-like extracellular matrix (ECM) has been described by workers in several laboratories (12–15). Recently, we have characterized the temporal pattern of expression of several genes encoding osteoblast phenotype proteins during a developmental sequence leading to an osteocyte-like cell in a mineralized ECM (16–19). These studies have defined three key time periods during the sequence. Initially, a burst of proliferative activity occurs, peaking between days 7 and 10, at which time cell growth genes (c-*myc* and c-*fos*) and cell cycle genes (core and H1 histone genes) are actively expressed (period I). It is during the proliferative period that expression of collagen genes results in production of the type I collagen ECM characteristic of osteoblasts (16, 20). With further ECM maturation, a decline in proliferation and the down-regulation of cell cycle- and cell growth-related genes occurs, at which time genes encoding alkaline phosphatase and osteopontin, noncollagenous osteoblast proteins, become expressed (period II). The ECM progressively takes on bone-like properties and then, with the onset of mineralization, there is increased expression of the noncollagenous ECM proteins, osteopontin and osteocalcin, and acceleration of an ordered deposition of calcium and phosphate. In well mineralized cultures (period III), day 23 and beyond, expression of alkaline phosphatase is down-regulated, while osteocalcin and osteopontin exhibit increased levels of expression, reflecting a later stage of osteoblast differentiation. This developmental sequence of gene expression in primary cultures of normal diploid calvarial osteoblasts is similar to that observed in calvaria *in vivo* (21).

Results presented in this paper show that the proteins of the nuclear matrix change in a differentiation stage-specific manner and thus provide markers for the progressive maturation of osteoblasts to osteocytes. These stage-specific changes in protein composition parallel modifications in gene expression. They are consistent with a functional role for components of the nuclear matrix in selectively and sequentially modulating expression of genes during development of the bone cell phenotype.

MATERIALS AND METHODS

Cell Culture. Timed pregnant rats (Sprague–Dawley) were obtained from Charles River Breeding Laboratories. The isolation and culture of primary osteoblasts were as described (16). Briefly, central bone of calvaria was dissected from gestational day 21 fetal pups and rinsed in sterile phosphate-buffered saline. The periosteum was first scraped off the bone and the calvaria were then subjected to three sequential trypsin/collagen digestions. After the third enzymatic digestion, the dissociated cells were passed through a sterile Swinex filter, pelleted by centrifugation, counted, and plated at a density of 5×10^5 cells per 100-mm culture plate. Cells were fed Eagle's minimal essential medium (MEM) supplemented with 10% fetal calf serum three times a week for the first 7 days after isolation. The cultures were then fed with BGJ_b medium supplemented with 10% fetal calf serum, 10

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Abbreviation: ECM, extracellular matrix.

mM β-glycerophosphate, and 50 μg of ascorbic acid per ml—conditions that support mineralization of the ECM. Subcultivation of primary cultures was performed by rinsing the cells with sterile phosphate-buffered saline and then adding 0.25% trypsin in Hanks' balanced salt solution without $CaCl_2$, $MgCl_2 \cdot 6H_2O$, and $MgSO_4 \cdot 7H_2O$ (GIBCO). The cells were incubated for 10 min at 37°C, then 5 ml of complete medium was added, and the cells were counted and plated at 5×10^5 per 100-mm culture dish.

Nuclear Matrix Protein Isolation. Two plates of cells on days 7, 11, 15, 19, and 23 were pulse-labeled with 25 μCi of L-[^{35}S]methionine per ml (New England Nuclear; 680 Ci/mmol; 1 Ci = 37 GBq) in methionine-free MEM for 2 hr at 37°C followed by the isolation of the nuclear matrix proteins according to the protocol of Fey *et al.* (22). The cells were rinsed first with phosphate-buffered saline and then lysed in cytoskeleton buffer (100 mM NaCl/300 mM sucrose/10 mM Pipes, pH 6.8/3 mM $MgCl_2$/0.5% Triton X-100/1.2 mM phenylmethylsulfonyl fluoride) for 5 min at 4°C. This step removes most of the soluble cytoplasmic and nuclear proteins, and the fidelity of the nuclear and cytoarchitecture is largely retained as determined by electron microscopy of both whole mounts and resinless sections of these preparations. The cells were further treated with a double detergent buffer (0.5% deoxycholate/1% Tween-40 in 10 mM NaCl/10 mM Tris·HCl/3 mM $MgCl_2$, pH 7.4), which removes the cytoskeleton and associated polyribosomes. Finally, the cells were digested with DNase I (100 μg/ml) and RNase A (50 μg/ml) in digestion buffer (same as cytoskeleton buffer but reduced to 50 mM NaCl) for 20 min at room temperature and then the chromatin-associated proteins were released by adding 2.0 M ammonium sulfate to a final concentration of 0.25 M. After centrifugation for 10 min at 1200 × *g*, the supernatant was discarded, and the pelleted nuclear matrix was solubilized in two-dimensional lysis buffer [9.5 M urea/2% ampholines (1.6% pH 5–7, 0.4% pH 3–10; Serva)/5% 2-mercaptoethanol/2% (wt/vol) Nonidet P-40] prior to electrophoresis. Two-dimensional gel electrophoresis was performed according to O'Farrell (23) and the resulting fluorograms were analyzed with an LKB scanning densitometer.

Biochemistry and Histochemistry. The following osteoblast parameters were assayed throughout the culture period. The osteocalcin secreted into the medium was measured by a radioimmunoassay as described (24). Autoradiography (25)

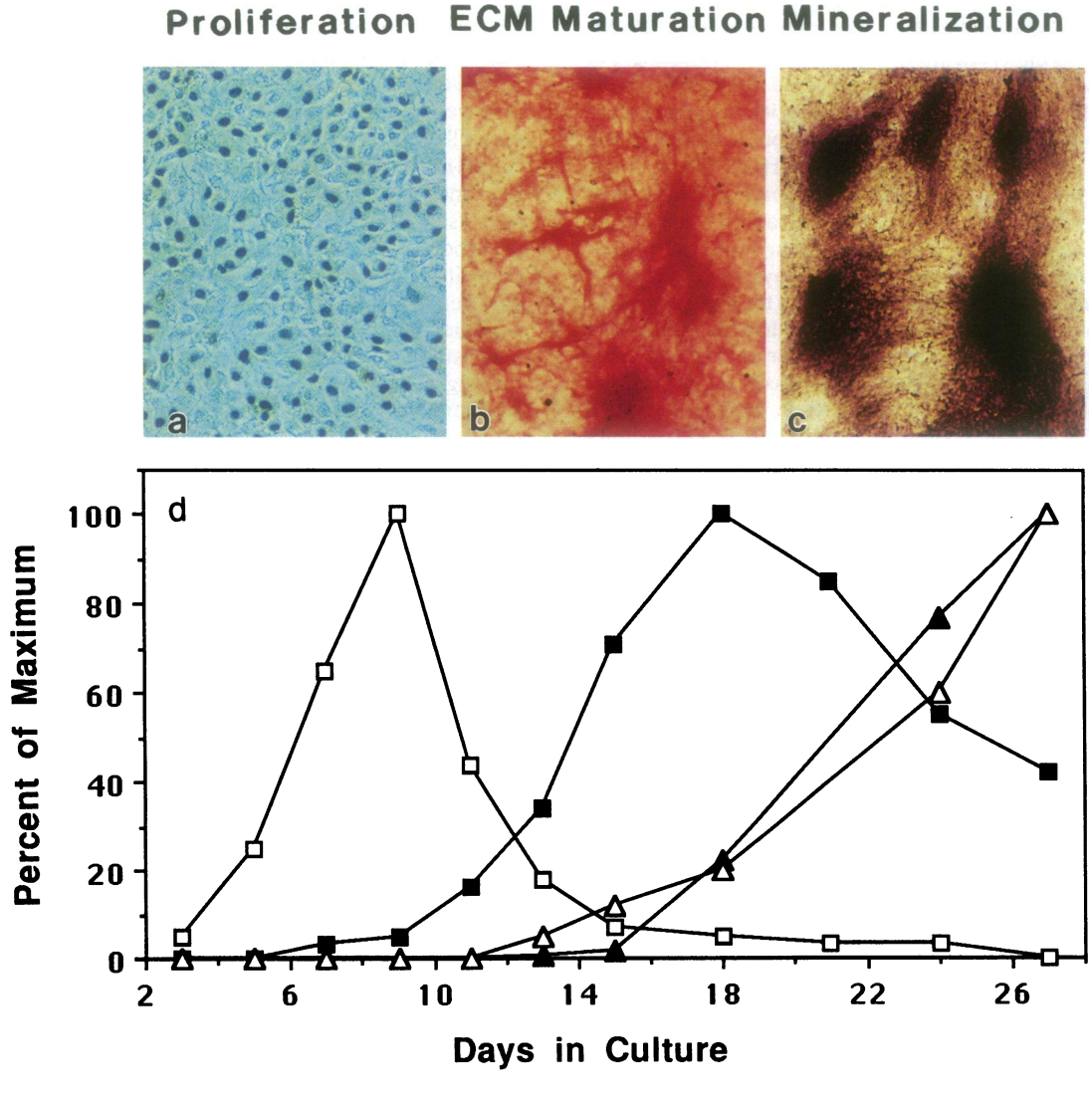

FIG. 1. Histochemistry and gene expression of primary rat osteoblasts. (*a*) Autoradiography shows that >95% of the cells incorporate [^{3}H]thymidine on day 7 (period I). These results correspond with maximal expression of the histone H4 gene (□) in *d* reflected by the mRNA levels during the proliferation period. (*b*) Maximal staining for alkaline phosphatase is observed at day 15 corresponding to high levels of alkaline phosphatase gene expression during period II (■ in *d*). Increased mRNA levels of osteocalcin (▲ in *d*) coincide with the period of ECM mineralization (period III) as shown by day 23 cultures staining for mineral deposition (*c*). Total calcium deposition (△ in *d*) (measured as μg per well) in the cell layer is also represented as percent of maximum.

was performed on [^{3}H]thymidine (20 Ci/mmol; Amersham)-labeled cells (1 μCi/ml) with Ilford K-5 photographic emulsion, which was exposed for 5–7 days at 4°C. Histochemical staining for alkaline phosphatase activity was performed by incubating the cells for 30 min at room temperature in 10 mM Tris·HCl (pH 8.4) containing 20 mg of disodium naphthol AS-MX phosphate per ml and 40 mg of Fast Red TR salt per ml (Sigma). Cultures were assessed for mineral deposition by von Kossa silver staining by incubating cell cultures in 3% $AgNO_3$ for 30 min. mRNA levels were determined by slot blot analysis as described (18, 26). Calcium concentrations in cell layers were determined by atomic adsorption after hydrolysis in 6 M HCl for 24 hr at 110°C. Alkaline phosphatase activity was measured by *p*-nitrophenol substrate reactions (16).

RESULTS

Histochemistry of the Culture System. Several approaches were used to monitor the stages of the *in vitro* culture system as it relates to development of the osteoblast phenotype. To determine the percentage of proliferating cells, *in situ* autoradiography was performed on [^{3}H]thymidine-labeled cells that were grown on coverslips. Fig. 1*a* shows that >95% of the cells on day 7 incorporated [^{3}H]thymidine as indicated by the presence of silver grains over the nuclei. These results correspond directly with histone gene expression (Fig. 1*d*), which is tightly coupled to DNA replication as reflected by the parallel relationship between H4 histone mRNA levels and DNA synthesis. After day 9, proliferation begins to decline and the ECM maturation period is initiated. This is visualized by staining cells for alkaline phosphatase. Maximal staining for alkaline phosphatase is observed at day 15 (Fig. 1*b*) and corresponds to the peak levels of alkaline phosphatase gene expression reflected by cellular levels of alkaline phosphatase mRNA (Fig. 1*d*). The final phase of the osteoblast developmental sequence is the mineralization of the ECM indicated by von Kossa silver staining, which occurs in the nodules of multilayered cells (Fig. 1*c*). The induction of osteocalcin gene expression coincides with this period of ECM mineralization (Fig. 1*d*).

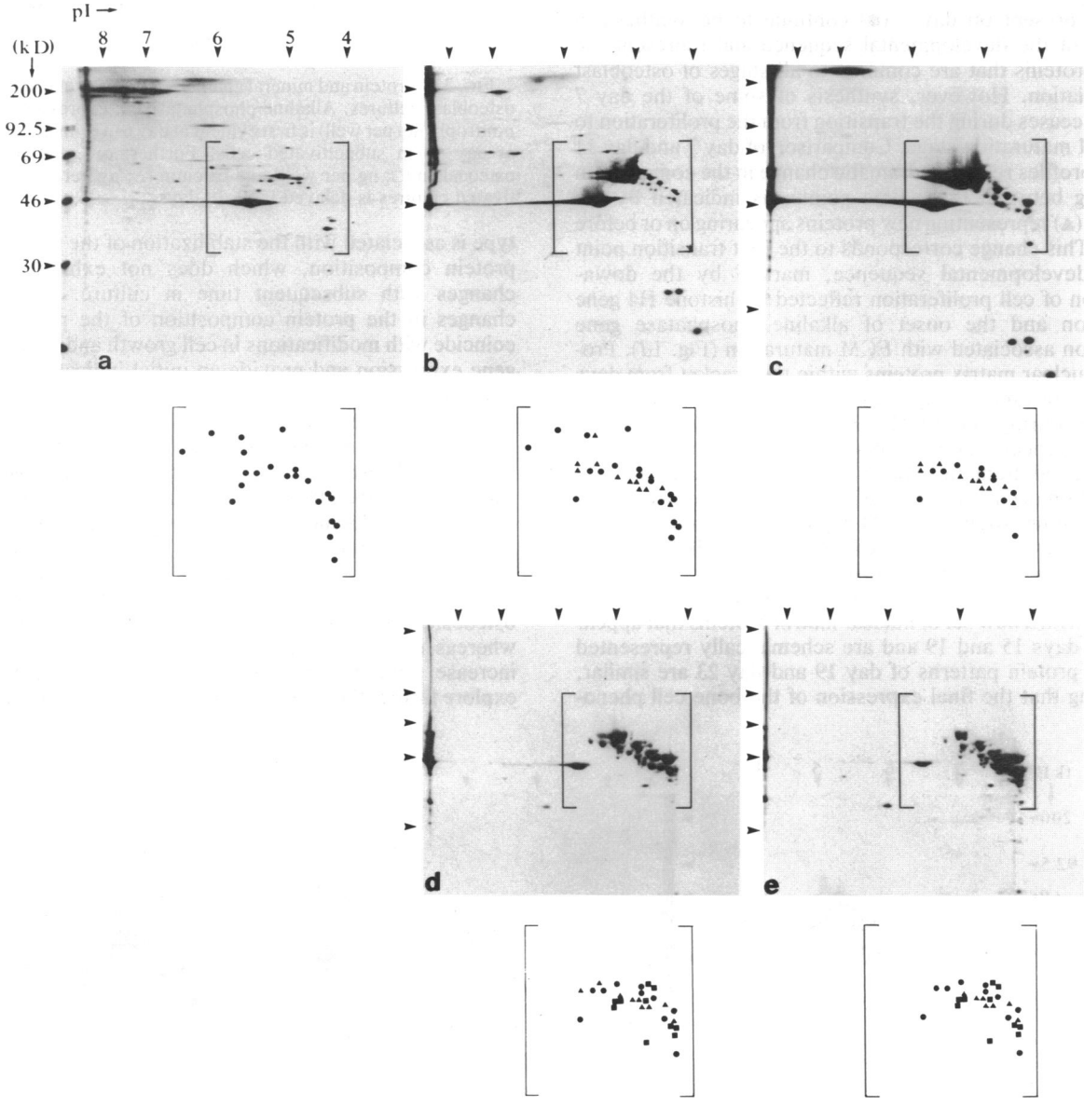

FIG. 2. Two-dimensional gel electrophoretic analysis of nuclear matrix proteins isolated from primary rat osteoblasts. Fluorographs of pulse-labeled nuclear matrix proteins isolated from cells within each period of osteoblast differentiation are shown with a schematic representation of proteins within the bracketed region of the gel. Symbols represent proteins that are synthesized within or before the following time periods: ●, day 7 (*a*); ▲, day 11 (*b*); ▲, day 15 (*c*); ■, day 19 (*d*); ■, day 23 (*e*).

Two-Dimensional Gel Electrophoretic Characterization of Nuclear Matrix Proteins During the Osteoblast Developmental Sequence. One-dimensional gel analysis of nuclear matrix proteins isolated from both proliferating and mineralized cell cultures revealed distinct differences in their protein composition (data not shown). To characterize these alterations in nuclear matrix protein expression, high-resolution two-dimensional gel electrophoresis was used to determine qualitative changes occurring within the three principal periods of the osteoblast developmental sequence and at the transition points between these stages.

The results from the two-dimensional gel analysis of nuclear matrix proteins from primary cultures on days 7 (proliferation), 11 and 15 (ECM maturation), and 19 and 23 (ECM mineralization) are shown in Fig. 2. A schematic representation of the protein pattern within the bracketed region is shown below each gel. The bracketed region was closely analyzed to determine the most significant changes that occurred in this area of the gel. The symbols in the schematics are used to designate the time during the culture period at which specific proteins are first observed. A number of proteins present on day 7 (●) continue to be synthesized throughout the developmental sequence and represent the matrix proteins that are common to all stages of osteoblast differentiation. However, synthesis of some of the day 7 proteins ceases during the transition from the proliferation to the ECM maturation stage. Comparison of day 7 and day 11 protein profiles reveals a dramatic change in the composition occurring between these time points, as indicated by the symbols (▲) representing new proteins appearing on or before day 11. This change corresponds to the first transition point of the developmental sequence, marked by the down-regulation of cell proliferation reflected by histone H4 gene expression and the onset of alkaline phosphatase gene expression associated with ECM maturation (Fig. 1*d*). Profiles of nuclear matrix proteins within the bracket from days 11 and 15 are similar, suggesting that only minor changes are occurring during the ECM maturation period. However, some differences can be observed elsewhere on the gel.

The second dramatic change in the nuclear matrix protein composition occurs at the time of the second developmental transition point when the ECM begins to mineralize. At this time, there is a decrease in cellular levels of alkaline phosphatase mRNA and an initial increase in osteocalcin gene expression (Fig. 1*d*). The initiation of mineralization is associated with a new set of nuclear matrix proteins that appear between days 15 and 19 and are schematically represented (■). The protein patterns of day 19 and day 23 are similar, suggesting that the final expression of the bone cell phenotype is associated with the stabilization of the nuclear matrix protein composition, which does not exhibit significant changes with subsequent time in culture. The dramatic changes in the protein composition of the nuclear matrix coincide with modifications in cell growth and tissue-specific gene expression and provide an initial indication of a functional association (coupling) of the nuclear matrix with modulation of gene expression during osteoblast development.

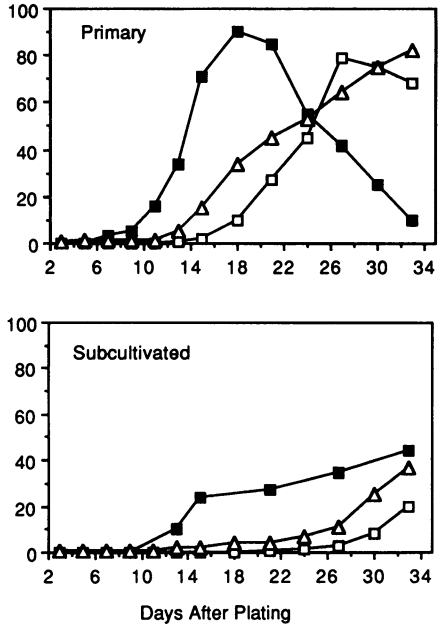

FIG. 3. Protein and mineral analysis of primary and subcultivated osteoblast cultures. Alkaline phosphatase (■; expressed as nmol of *p*-nitrophenol per well) is first evident at day 6 in primary cultures and at day 10 in subcultivated cells. Furthermore, the presence of osteocalcin (□; ng per well) and calcium (△; μg per well) in subcultivated cultures is delayed by 9–10 days.

Preservation of Stage-Specific Nuclear Matrix Protein Expression When the Osteoblast Developmental Sequence Is Altered. The subcultivation of primary rat osteoblast cultures delays the developmental expression of the mature osteoblast phenotype. As shown in Fig. 3, the initial increase in expression of alkaline phosphatase is shifted from day 9 in the primary cultures to day 12 in the subcultivated cells. The osteocalcin level in primary cell cultures peaks at day 27, whereas in subcultivated cells the level does not begin to increase until day 23. These observations were used to explore further the extent to which changes in nuclear matrix

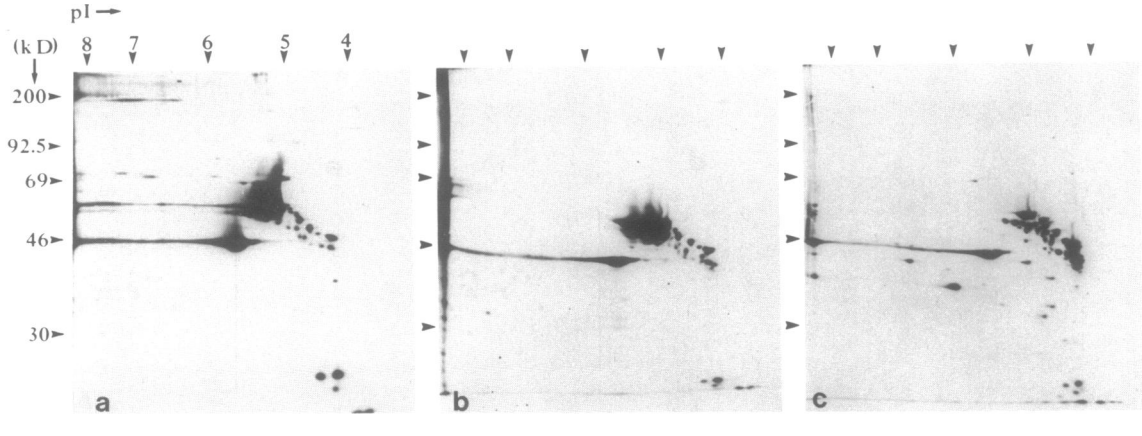

FIG. 4. Two-dimensional gel electrophoretic analysis of nuclear matrix proteins isolated from subcultivated osteoblasts. Fluorographs of pulse-labeled nuclear matrix proteins isolated from day 8 (*a*), day 26 (*b*), and day 33 (*c*) cultures of subcultivated osteoblasts. The appearance of the mineralized nuclear matrix protein profile from primary cultures (day 23 in Fig. 2*e*) does not appear until day 33 of the subcultivated cells.

protein profiles reflect stage-specific differentiation and to establish that such changes are not related solely to the duration of the culture period. The two-dimensional gel patterns of nuclear matrix proteins isolated from primary and passaged cultures were compared. Fig. 4 shows the nuclear matrix protein patterns from days 8, 26, and 33 of passaged cells. Nuclear matrices isolated from days 16 and 20 are not shown, as they are identical to the day 26 profile; similarly, day 12 was comparable to day 8. Comparison of Figs. 2*e* and 4*c* indicates that the primary cell nuclear matrix pattern characteristic of the mineralized phenotype (day 23) does not appear until day 33 of the subcultivated cells. Thus, the delayed course of gene expression in the subcultivated cell cultures is accompanied by a similar change in nuclear matrix protein patterns. There appears to be a stringent relationship between the specific period of the developmental sequence and the pattern of the nuclear matrix proteins. This relationship is also indicated by the corresponding delay from day 15 to day 26 of the appearance of the matrix protein profile characteristic of the ECM maturation period in the passaged cells, which reflects the delay in the onset of tissue-specific gene expression. The extended ECM maturation period in the passaged cells is accompanied by persistence of the associated nuclear matrix profiles on days 16, 20, and 26. This result suggests that the ECM maturation period is prolonged until a competent ECM is developed to support entry into the mineralization phase of the differentiation sequence.

CONCLUSIONS

The results presented here indicate that the protein composition of the nuclear matrix is an effective marker for identifying specific stages of osteoblast differentiation. The data are consistent with a role for the nuclear matrix in supporting and/or regulating expression of genes during the progression of osteoblast differentiation. This role is suggested by the correlation of nuclear matrix patterns with each of the three principal periods of the osteoblast developmental sequence and the persistence of these patterns with only minor variations within each period. Also there are pronounced changes in the nuclear matrix protein patterns at the time of the two major transition points in the osteoblast developmental sequence. This is when modifications in the sequential and selective expression of cell growth and tissue-specific genes are observed. This relationship between nuclear matrix protein composition and osteoblast stage-specific gene expression is maintained even when the onset of ECM mineralization is delayed by subcultivating primary cultures of calvarial osteoblasts. Taken together, the evidence is consistent with a role of the nuclear matrix in mediating the gene expression associated with the progressive acquisition of the bone cell phenotype.

Specific mechanisms by which the nuclear matrix may mediate cell- and tissue-specific gene expression remain to be established. Our results suggest that there are two primary classes of nuclear matrix proteins: (*i*) "common" proteins, whose synthesis persists throughout the development of the osteoblast phenotype; and (*ii*) proteins that are specific to a given stage of osteoblast differentiation. The nuclear matrix may be composed of proteins that are restricted to a specific stage of bone cell differentiation and of those required for general metabolic processes. One can speculate that the nuclear matrix proteins may be involved with the localization and/or organization of specific genes as well as serving to concentrate and/or facilitate the interactions of promoter-binding factors with regulatory elements of genes that are actively transcribed.

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