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Association between the expression of the c-*myc* oncogene mRNA and the expression of the receptor protein for 1,25-dihydroxyvitamin D_3

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ABSTRACT **Studies in lymphocytes have indicated similarities in the state of activation, the time kinetics, and the pathologic states associated with the expression of the c-*myc* oncogene, and the expression of the 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$] receptor protein. Here, we have sought evidence for an association between c-myc and the 1,25-$(OH)_2D_3$ receptor protein in mammalian cells other than lymphocytes. Comparing two rat osteogenic sarcoma cell lines, one that produces constitutively relatively high levels of the 1,25-$(OH)_2D_3$ receptor protein (ROS 17/2.8) and one in which the 1,25-$(OH)_2D_3$ receptor protein is practically undetectable (ROS 2/3), we found that the 1,25-$(OH)_2D_3$ receptor-expressing cell line also expressed c-myc mRNA. In contrast, the cell line in which the 1,25-$(OH)_2D_3$ receptor was undetectable did not express c-myc mRNA. Furthermore, we transfected mouse skin fibroblasts (NIH 3T3) with a recombinant plasmid carrying the human c-*myc* oncogene. We found a dramatic increase in the 1,25-$(OH)_2D_3$ receptor concentration in five separate clonal lines of NIH 3T3 cells transfected with the c-*myc*-carrying plasmid compared to their nontransfected counterparts or to NIH 3T3 fibroblasts transfected with the vector plasmid alone. The receptor protein of the transfected cells exhibited biochemical characteristics indistinguishable from those of classical receptors for 1,25-$(OH)_2D_3$. The increased expression in the transfected cells appeared specific for the receptor for 1,25-$(OH)_2D_3$; receptors for sex steroids were not detected in the nontransfected NIH 3T3 cells and remained undetectable after transfection with c-*myc*. Moreover, the level of the glucocorticoid receptor protein, which was expressed in the nontransfected cells, did not change upon transfection with c-*myc*.**

Structural homologies between the oncogene v-*erbA* and the genes encoding the receptor proteins for glucocorticoids and for estrogens have been described recently (1, 2). Similar to these classical steroid hormones, the hormonal form of vitamin D_3—namely 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$]—mediates its biological effects by binding to a well-characterized nuclear receptor (3). The 1,25-$(OH)_2D_3$ receptor protein has a molecular weight of 52,000–60,000, depending on the species, and it is well preserved throughout evolution (4). Over the past few years, a most interesting finding in the 1,25-$(OH)_2D_3$ receptor field has been the ubiquity of this protein in a large variety of mammalian cells (5). We and others have found that activation of both T and B lymphocytes with mitogenic lectins, antigens, or viruses leads to the expression of the receptor protein for 1,25-$(OH)_2D_3$ (6–9). In T lymphocytes, the 1,25-$(OH)_2D_3$ receptor becomes detectable within 12 hr of activation with lectins, its concentration reaches a peak at the G_{1a} phase of the cell cycle, and it declines when the lymphocytes enter the S phase of the cycle (9). Using lymphocyte preparations from animal thymus as well as from human thymus and tonsils, we have observed a strong positive correlation between the concentration of the 1,25-$(OH)_2D_3$ receptor sites in lymphocytes (without prior *in vitro* activation) and the number of activated cells (10). Furthermore, we have found that peripheral blood lymphocytes from patients with rheumatoid arthritis express the 1,25-$(OH)_2D_3$ receptor protein (without *in vitro* activation) with high frequency, in contrast to lymphocytes of normal individuals (11).

It is well established that activation of both T and B lymphocytes involves the expression of oncogenes (12–14). Specifically, the nuclear oncogenes c-*fos*, c-*myc*, and c-*myb* are sequentially expressed upon activation of lymphocytes. In T cells, an increase in c-myc mRNA expression occurs at ≈1.5 hr after activation, reaches a maximum at 5 hr, and persists for at least 18 hr. Expression of c-fos mRNA, on the other hand, occurs within minutes after stimulation, but decreases within 1.5 hr. Finally, c-myb mRNA expression increases in activated T cells but not in activated B cells (14). In addition, lymphocytes from patients with autoimmune disorders, including rheumatoid arthritis, exhibit increased c-myc mRNA expression compared to lymphocytes from normal controls (14).

Prompted by the apparent similarities in the state of activation, the time kinetics, and the pathologic situations associated with the expression of c-myc mRNA and the expression of the 1,25-$(OH)_2D_3$ receptor protein in lymphocytes, we have examined here whether the association between the expression of c-myc and the expression of the 1,25-$(OH)_2D_3$ receptor protein is a phenomenon occurring in mammalian cells other than lymphocytes. In these studies, we used two established lines of rat osteogenic sarcoma cells, one that expresses constitutively high levels of the 1,25-$(OH)_2D_3$ receptor protein (ROS 17/2.8) and one in which this receptor protein is virtually undetectable (ROS 2/3) (15). We compared c-myc RNA expression between these cells. In addition, we transfected mouse skin fibroblasts (NIH 3T3) with a recombinant plasmid carrying the human c-*myc* gene.¶ We then compared the level of expression of the 1,25-$(OH)_2D_3$ receptor protein, as well as the expression of other steroid hormone receptors, between nontransfected and c-*myc*-transfected cells.

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Abbreviation: 1,25-$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3.

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¶The recommended designation for this gene is *MYC* (16).

EXPERIMENTAL PROCEDURES

Materials. 1,25-$(OH)_2$[26,27-^{3}H]D_3 (158 Ci/mmol; 1 Ci = 37 GBq), [1,2,4-^{3}H]triamcinolone acetonide (30 Ci/mmol), [2,4,6,7-^{3}H]estradiol (101 Ci/mmol), and [1,2,6,7-^{3}H]testosterone (88 Ci/mmol) were purchased from Amersham. Crystalline 1,25-$(OH)_2D_3$ was a generous gift from Milan Uskokovic (Hoffmann–La Roche). A complementary DNA probe (third exon) of the human c-*myc* oncogene was purchased from Oncor (Gaithersburg, MD). The rat osteogenic sarcoma cell lines ROS 17/2.8 and ROS 2/3 were maintained in our laboratory as described in detail (15).

Isolation of RNA. Total cellular RNA was isolated from confluent monolayer cultures of ROS cells by the guanidinium isothiocyanate/cesium chloride centrifugation method (17). The cell pellets were solubilized and the extract was overlaid on a 1.2-ml cushion of 5.7 M CsCl in 0.1 M EDTA (pH 7.5). After centrifugation at 114,000 × *g* for 22 hr at 20°C, the resulting RNA pellet was dissolved in 10 mM Tris·HCl, pH 7.4/5 mM EDTA/1% $NaDodSO_4$, extracted once with chloroform/butanol (4:1, vol/vol), twice with chloroform/phenol (1:1), and then repeatedly precipitated with ethanol until an absorbance ratio (A_{260}/A_{280}) of 2.0 was obtained. The quality of the product was tested by agarose gel electrophoresis followed by ethidium bromide staining, or by transfer to nitrocellulose paper and methylene blue staining.

Hybridization with a c-myc cDNA Probe. The total cellular RNA was separated by 1% agarose gel electrophoresis in the presence of 6% formaldehyde (17), transferred to nitrocellulose, and hybridized with a ^{32}P-labeled αdCTP cDNA probe (third exon) for human c-*myc* (Oncor). The preparation of the filter, the transfer to nitrocellulose, and the hybridization were conducted according to the procedures described by Meinkoth and Wahl (18). The first wash of the filter was conducted in 2× SSC/0.1% $NaDodSO_4$ (1× SSC = 0.15 M NaCl/0.015 M sodium citrate) for 30 min at 22°C and the final wash was in 0.2× SSC at 42°C for 15 min. *Hae* III digest of φX174 replicative form DNA molecular weight standards (IBI, New Haven, CT) were run in parallel and used to estimate the molecular weight of the RNA bands.

Plasmids and Transfection. Plasmid pMCGM1 carrying the normal human c-*myc* gene, the aminoglycoside phosphotransferase gene, and the Moloney murine sarcoma virus (Mo-MSV) enhancer was constructed as described in detail elsewhere (19) by inserting a 2.9-kilobase (kb) *Eco*RI fragment carrying the aminoglycoside phosphotransferase gene under the 5′ transcriptional control sequences of Mo-MSV long terminal repeat and the 3′ polyadenylylation signals of the herpes simplex virus type 1 thymidine kinase gene into the single *Eco*RI site of plasmid pMC41/C1. Transfection of the mouse skin fibroblast line NIH 3T3 with plasmid pMCGM1 was accomplished by a modification (20) of the calcium phosphate technique (21) and selection of clones in medium containing 200 μg of geneticin per ml as described earlier (22).

Receptor Binding Studies. Cells were incubated intact either with 1,25-$(OH)_2$[^{3}H]D_3 (2 nM) for 1 hr at 37°C in the presence or absence of a 200-fold excess of unlabeled 1,25-$(OH)_2D_3$, or with [^{3}H]testosterone, [^{3}H]estradiol, and [^{3}H]triamcinolone acetonide (2 nM) alone. Cells were then cooled at 4°C for 15 min, washed with isotonic buffer, and disrupted by sonication. Cytosol was prepared by ultracentrifugation (105,000 × *g*) of the sonicate and was either layered onto sucrose density gradients (4–24% in 0.3 M KCl buffer) or precipitated by ammonium sulfate, resolubilized, and applied to a DNA-cellulose column. The column was then eluted with a 90-ml linear gradient of KCl (0–0.6 M KCl). Fractions were collected and aliquots (1 ml) were counted. Salt concentration was monitored by conductivity measurements. For the saturation analysis of the 1,25-$(OH)_2$[^{3}H]D_3 binding, cells were incubated as described above with increasing concentrations of 1,25-$(OH)_2$[^{3}H]D_3 (30 pM to 1 nM) alone or in the presence of a 200-fold excess of unlabeled 1,25-$(OH)_2D_3$. After incubation, cells were washed and solubilized with Omnisol (WestChem, San Diego, CA), and the internalized radioactivity was counted. The specific binding (total minus nonspecific) was plotted by the method of Scatchard. The above procedures have been described in detail in earlier publications (6, 15, 23).

RESULTS

The results of the experiments in which we compared the expression of c-myc mRNA and the expression of the 1,25-$(OH)_2D_3$ receptor protein in the two rat osteogenic sarcoma cell lines are illustrated in Fig. 1.

RNA analysis of total RNA with a human c-*myc* probe is shown (Fig. 1 *Upper*). In the RNA preparation of the ROS 17/2.8 cells, ^{32}P-labeled c-*myc* probe hybridized with three distinct bands; one of 2.5 kb, one of 2.2 kb, and a third and larger one of 1.8 kb. Sequence homology of the *myc* gene among species, including human and rat, has been described (24). In contrast to the ROS 17/2.8 cells, the RNA preparation of the ROS 2/3 cells failed to show any bands hybridizing with the probe. Scatchard analysis of the specific binding of 1,25-$(OH)_2$[^{3}H]D_3 in these two cell lines is shown in Fig. 1 (*Lower*). In agreement with earlier findings of ours (15), the ROS 17/2.8 cells exhibited high-affinity binding (K_d, 0.9 nM) and a maximal concentration of binding sites of ≈25 fmol per 1 × 10^6 cells. In contrast, the ROS 2/3 exhibited only nonspecific binding to 1,25-$(OH)_2$[^{3}H]D_3.

More direct evidence for the association between c-*myc* and the expression of 1,25-$(OH)_2D_3$ receptor was derived from the results of the experiment shown in Fig. 2. Scatchard analysis of the specific binding of 1,25-$(OH)_2$[^{3}H]D_3 in clone

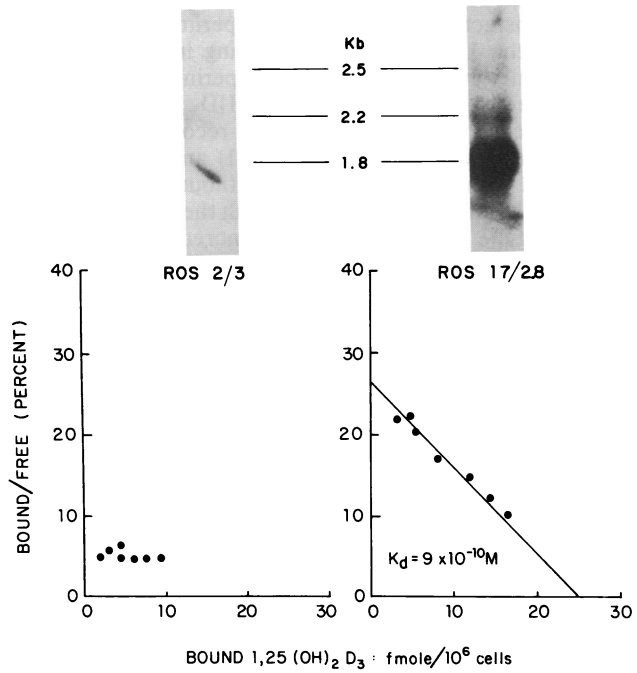

FIG. 1. Comparison of c-*myc* expression and 1,25-$(OH)_2$[^{3}H]D_3 binding in two rat osteogenic sarcoma cell lines. (*Upper*) Results of RNA blot analysis of total cellular RNA from ROS 2/3 (*Left*) and ROS 17/2.8 (*Right*) rat osteogenic sarcoma cells hybridized with a ^{32}P-labeled human cDNA probe (third exon) of the c-*myc* gene. The size of the hybridized bands in the ROS 17/2.8 cells (2.5, 2.2, and 1.8 kb) was determined by comparison with DNA standards. (*Lower*) Scatchard analysis of the 1,25-$(OH)_2$[^{3}H]D_3 binding in these two cell lines.

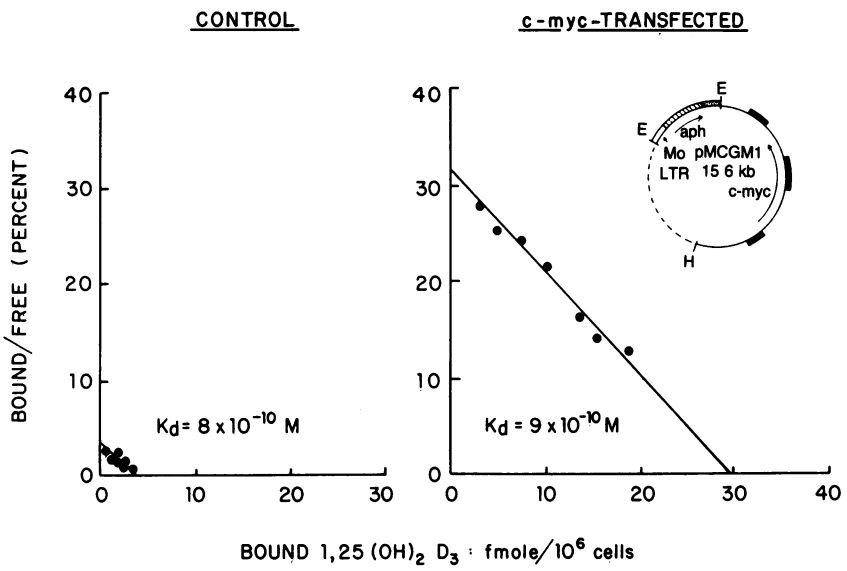

FIG. 2. Scatchard analysis of 1,25-$(OH)_2[^3H]$-D_3 binding in NIH 3T3 mouse skin fibroblasts (control) and in the clonal line MN-1 of NIH 3T3 cells transfected with a recombinant plasmid carrying the human c-*myc* gene (*Inset*). LTR, long terminal repeat; aph, aminoglycoside phosphotransferase.

MN-1 of c-*myc*-transfected NIH 3T3 cells (*Right*) revealed a 10-fold higher concentration of binding sites than the nontransfected NIH 3T3 cells grown under identical conditions. An increase of the 1,25-$(OH)_2[^3H]D_3$ receptor binding in this clone of c-*myc*-transfected fibroblasts compared to the nontransfected controls was found in four additional experiments in which we quantitated the binding either by means of saturation analysis or by calculating the amount of radioactivity bound on the sedimenting peak in sucrose gradients. The extent of the increase in the 1,25-$(OH)_2[^3H]D_3$ binding between the MN-1 clone of the transfected cells and the nontransfected fibroblasts, however, varied from a maximum of 10-fold to a minimum of 2-fold. This variation was primarily due to changes of the 1,25-$(OH)_2[^3H]D_3$ binding in the nontransfected cells; in all five experiments the concentration of the 1,25-$(OH)_2[^3H]D_3$ binding in the transfected cells remained very similar. In two experiments (not shown) we compared also the 1,25-$(OH)_2[^3H]D_3$ binding between NIH 3T3 cells transfected with the recombinant plasmid carrying the c-*myc* gene (clone MN-1) and NIH 3T3 cells transfected with the same plasmid without the c-*myc* gene. We found that the cells transfected with the plasmid carrying the *myc* gene exhibited ≈2-fold increase in the 1,25-$(OH)_2[^3H]D_3$ binding compared to cells transfected with the vector alone. To exclude the possibility that the increase in the 1,25-$(OH)_2D_3$ receptor upon c-*myc* transfection was limited to one clonal line (MN-1) of the NIH 3T3 fibroblasts and thus could be due to selection of cells with coincidental greater expression of the 1,25-$(OH)_2D_3$ receptor for reasons other than the c-*myc* transfection, we examined four additional clonal lines of c-*myc*-transfected NIH 3T3 cells (MN-2, MN-3, MN-4, and MN-5). Fig. 3 illustrates comparison of 1,25-$(OH)_2[^3H]D_3$ binding (determined by sucrose density gradient sedimentation) among nontransfected, vector-transfected, and the four additional clonal lines of the c-*myc*-transfected NIH 3T3 cells. As in the experiments described above, we found no difference between nontransfected and vector-transfected cells. Nevertheless, all four c-*myc*-transfected clonal lines exhibited an increase in 1,25-$(OH)_2[^3H]D_3$ binding ranging from 1.3- to 3.1-fold compared to the nontransfected cells.

Fig. 4 illustrates the biochemical characterization of the 1,25-$(OH)_2[^3H]D_3$ binding in the MN-1 clone of the c-*myc*-transfected NIH 3T3 fibroblasts. The bound radioactivity in the transfected cells sedimented at a single peak at 3.3 S in sucrose density gradients and could be displaced by a 200-fold excess of unlabeled 1,25-$(OH)_2D_3$. In addition, the 1,25-$(OH)_2[^3H]D_3$-binding protein complex bound to DNA-cellulose and was eluted from this affinity resin with a gradient of KCl; the peak of the radioactivity was eluted at 0.3 M KCl. These properties are indistinguishable from the properties of classical 1,25-$(OH)_2D_3$ receptors.

To examine whether the association between c-*myc* transfection and the increase in the 1,25-$(OH)_2D_3$ receptor binding in NIH 3T3 cells was specific for this steroid hormone receptor protein and not for other steroid hormone receptors, we compared the binding of $[^3H]$testosterone, $[^3H]$estradiol, and $[^3H]$triamcinolone acetonide between the c-*myc*-transfected (MN-1) and nontransfected cells (Fig. 5). Transfected and nontransfected NIH 3T3 cells showed no evidence of

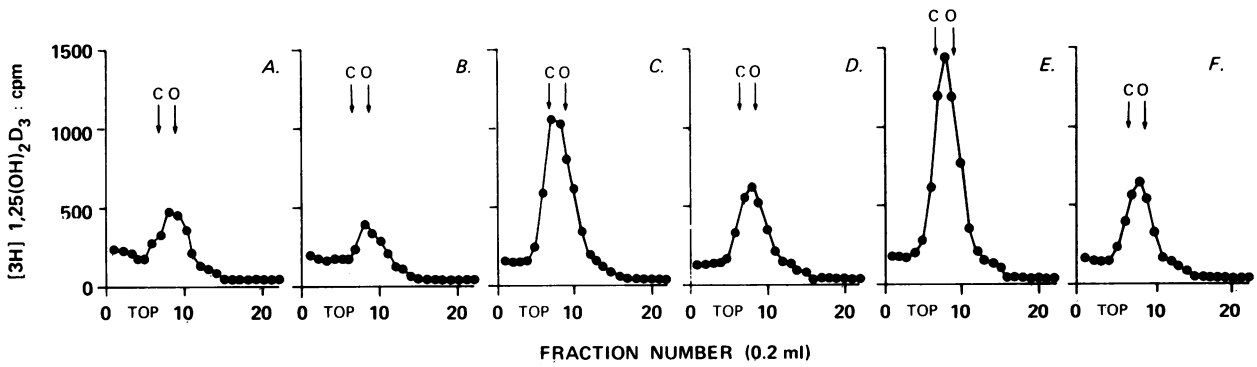

FIG. 3. Sucrose density gradient (4–24%) analysis of 1,25-$(OH)_2[^3H]D_3$ binding in nontransfected NIH 3T3 cells (*A*), NIH 3T3 cells transfected with vector plasmid alone (*B*), and four clonal lines (MN-2, MN-3, MN-4, and MN-5) of NIH 3T3 transfected with the vector carrying the c-*myc* gene (*C–F*). Arrows indicate the sedimentation position of markers. C, chymotrypsinogen (S = 2.54); O, ovalbumin (S = 3.7).

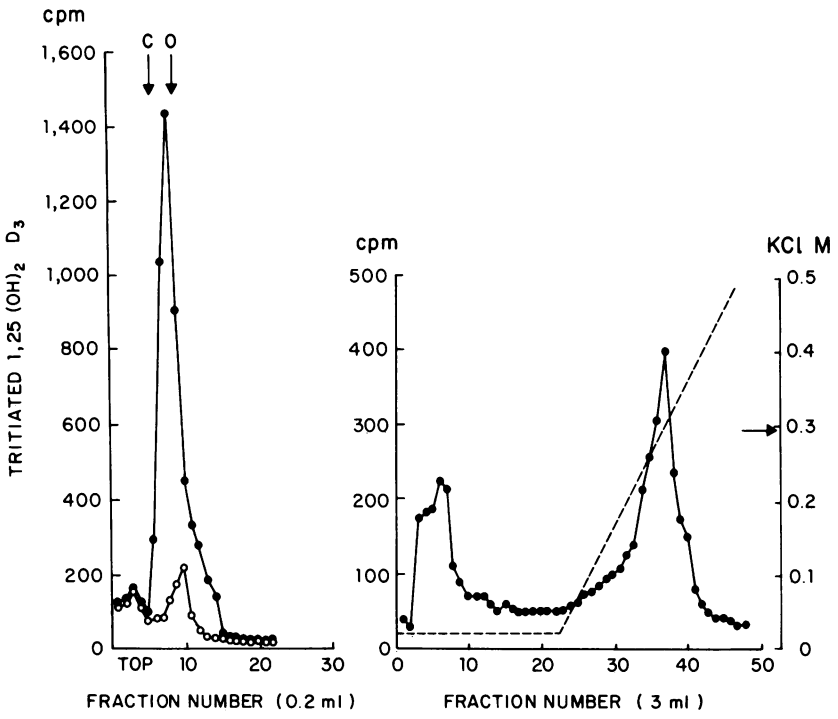

FIG. 4. Biochemical characterization of the 1,25-$(OH)_2[^3H]D_3$ binding in NIH 3T3 fibroblasts transfected with the c-*myc* gene (clone MN-1). (*Left*) Sucrose gradient (4–24%) analysis of the binding in cells incubated with either the labeled ligand (2 nM) alone (●) or in the presence of a 200-fold molar excess of unlabeled 1,25-$(OH)_2D_3$ (○). Arrows indicate the sedimentation position of markers. C, chymotrypsinogen (S = 2.54); O, ovalbumin (S = 3.7). (*Right*) Elution profile of the 1,25-$(OH)_2[^3H]D_3$ binding (●) from DNA cellulose during a KCl gradient. The salt concentration is depicted by the dashed line (– – –). Arrow indicates the concentration of KCl corresponding with the peak of the eluted radioactivity.

receptor-like binding for the sex steroids, but they exhibited receptor-like binding for the glucocorticosteroid. Nevertheless, there was no appreciable change in the amount of radioactivity bound to the glucocorticoid receptor between the transfected and the nontransfected cells.

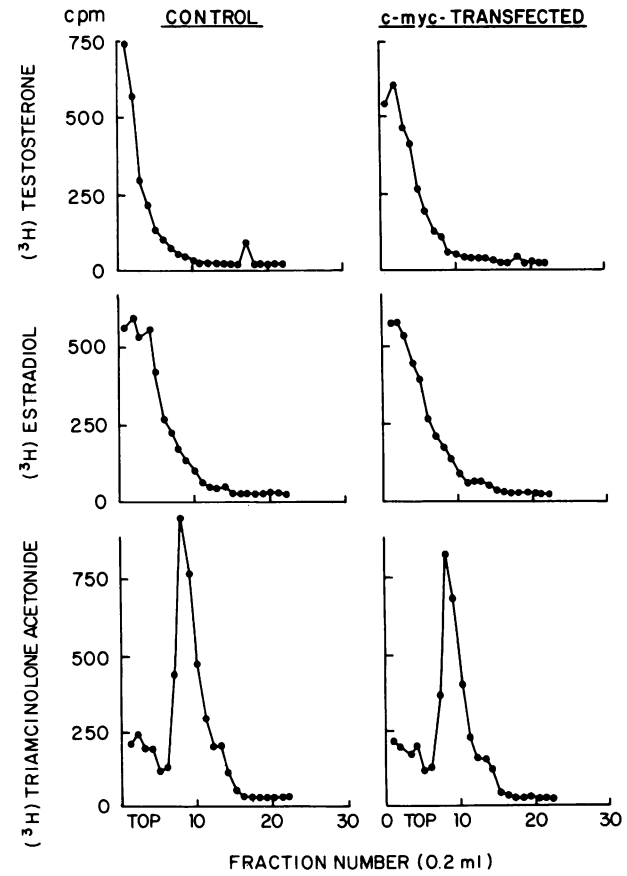

FIG. 5. Sucrose density gradient (4–24%) analysis of ^{3}H-labeled testosterone, estradiol, and triamcinolone acetonide in NIH 3T3 cells (*Left*) and the MN-1 clone of NIH 3T3 cells transfected with the recombinant plasmid carrying the c-*myc* gene (*Right*).

DISCUSSION

Earlier findings from studies on the expression of the receptor protein for 1,25-$(OH)_2D_3$ in activated T and B lymphocytes (6–11) taken together with findings from studies on the expression of the c-*myc* protooncogene in these cells (12–14, 24) have suggested to us that there might be an association between the two events. In the present study, we have probed into this putative association and sought evidence for a correlation between c-myc mRNA expression and the expression of the 1,25-$(OH)_2D_3$ receptor protein in eukaryotic cells other than lymphocytes.

We have found that the rat osteogenic sarcoma cell line ROS 17/2.8, which expressed relatively high levels of the 1,25-$(OH)_2D_3$ receptor protein, also expressed high levels of c-myc mRNA. In contrast, the rat osteogenic sarcoma line ROS 2/3, in which the 1,25-$(OH)_2D_3$ receptor protein was undetectable, did not express c-myc mRNA either. Furthermore, we transfected mouse skin fibroblasts (NIH 3T3), which express relatively low levels of the 1,25-$(OH)_2D_3$ receptor protein as well as of the c-myc mRNA, with a recombinant plasmid carrying the human c-*myc* oncogene (19). In five different clones of the transfected cells that we examined, we detected an increase in the 1,25-$(OH)_2D_3$ receptor binding in the c-*myc*-transfected cells compared to nontransfected NIH 3T3 cells or NIH 3T3 cells transfected with the plasmid without the c-*myc* gene. The biochemical characteristics of the receptor protein in the transfected cells were indistinguishable from those of classical receptors for 1,25-$(OH)_2D_3$. In our studies, we observed significant variation in the level of 1,25-$(OH)_2D_3$ binding in the nontransfected cells. Changes of the 1,25-$(OH)_2D_3$ receptor concentration at different stages of the culture have been reported earlier by us and others in primary cell cultures and in established cell lines (25, 26). It is possible that this phenomenon might be related to either the asynchronous nature of the cultures and/or to varying influence of factors present in the serum (i.e., platelet-derived growth factor) that affect expression of c-*myc* (12).

Because of the evidence that the receptor proteins for sex steroids were not detected in the nontransfected cells and remained undetectable after transfection with c-*myc* and that the level of the glucocorticoid receptor protein did not change

upon transfection with c-*myc*, we tentatively conclude that the increased expression of the 1,25-$(OH)_2D_3$ receptor protein in the transfected cells was specific for the receptor for this hormone and not for other steroid hormone receptors.

The findings of this study add support to the possibility that there might exist an association between c-myc mRNA expression and the expression of the 1,25-$(OH)_2D_3$ receptor protein in eukaryotic cells at large. The evidence at this stage, however, is only circumstantial and the molecular basis of this association is only a matter of conjecture. In experiments not shown here, we found no difference in the 1,25-$(OH)_2D_3$ receptor protein expression between c-*ras*-transfected NIH 3T3 fibroblasts compared to nontransfected control cells, suggesting that the association between c-myc mRNA expression and the expression of the 1,25-$(OH)_2D_3$ receptor is perhaps limited to this oncogene rather than being a general phenomenon associated with oncogene transfection in general. Also from preliminary experiments of ours not shown here, we have obtained evidence against the possibility that the c-*myc*-encoded protein is structurally similar to the 1,25-$(OH)_2D_3$ receptor protein. Indeed, semi-purified c-myc protein (generous gift from R. J. Fisher and T. S. Papas from the National Cancer Institute) did not bind 1,25-$(OH)_2[^3H]D_3$, and an antibody raised against the c-myc protein failed to recognize the 1,25-$(OH)_2D_3$ receptor protein. This evidence is consistent with the recent unpublished report for a structural homology of the NH_2 terminus of the 1,25-$(OH)_2D_3$ receptor gene with the v-*erbA* gene but not with the c-*myc* gene.[‖]

1,25-$(OH)_2D_3$, acting via its receptor protein, has been shown to be a potent regulator of the differentiation and proliferation of a variety of cell types, including normal and malignant blood monocytic cells as well as fibroblasts and osteoblast-like rat osteogenic sarcoma cells (27–30). Phenotypic changes induced by 1,25-$(OH)_2D_3$ in the promyelocytic leukemia cell line HL-60 were shown to be preceded by a marked decrease in the expression of the c-*myc* oncogene (31). 1,25-$(OH)_2D_3$ was also shown to inhibit the proliferation of activated lymphocytes (32) by blocking these cells at the G_{1a}–G_{1b} border of the cell cycle (9). Other inhibitors of lymphocyte proliferation such as cyclosporin A, dexamethasone, and the OKT11A antibody seem to exert their antiproliferative effect by decreasing the levels of c-myc mRNA at various points of the G_1 phase of the lymphocyte cycle (33). In view of this, it is likely that the antiproliferative effect of 1,25-$(OH)_2D_3$ on lymphocytes might be exerted also through influence of this hormone on c-myc transcription. The protein encoded by the c-*myc* gene is thought to perturb the activity or the specificity of the cellular transcription apparatus and thus mobilize cellular genes whose products are critical to growth and differentiation (34). In view of the above considerations and the evidence presented in this study, we are tempted to speculate that 1,25-$(OH)_2D_3$, acting via its receptor protein, might be a natural counterregulatory signal in oncogene-driven cell proliferation and differentiation.

Further work aiming to establish the association suggested by the results of the present study and its mechanistic basis might help to explain the findings of the ubiquity of the 1,25-$(OH)_2D_3$ receptor in a plethora of previously unsuspected cell types (5). Such knowledge should put into perspective the classical role of 1,25-$(OH)_2D_3$ in mineral homeostasis versus its wider biologic role in cell proliferation and differentiation at large. That is to say that the 1,25-$(OH)_2D_3$ receptor proteins, which are expressed constitutively in the limited number of the classical target tissues of the hormone (intestine, bone, and kidney), might be responsible for the well-established role of 1,25-$(OH)_2D_3$ in mineral and skeletal homeostasis. In contrast, the receptor proteins that are expressed in practically every cell type, perhaps as the result of oncogene activation, might be responsible for the recently appreciated and widespread involvement of 1,25-$(OH)_2D_3$ in the regulation of cell growth and differentiation.

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