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Alteration of Homeobox Gene Expression by N-*ras* Transformation of PA-1 Human Teratocarcinoma Cells

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We used a series of cell clones from a human teratocarcinoma cell line, PA-1, to study the effect of transformation by an activated N-*ras* oncogene on the expression of genes involved in retinoic acid (RA)-induced differentiation and growth regulation. Recently, it has been shown that expression of human HOX 2 genes is sequentially activated by RA beginning from Hox 2.9 at the 3′ end of the HOX 2 cluster (A. Simeone, D. Acampora, L. Arcioni, P. W. Andrews, E. Boncinelli, and F. Mavilio, Nature [London] 346:763–766, 1990). We now report that six different genes of the cluster HOX 1 are sequentially induced by RA in a similar temporal pattern, beginning with genes at the 3′ end of the cluster. However, in N-*ras*-transformed cell clones, RA-induced expression of these homeobox genes is delayed. Hox 1.4 and Hox 1.3, genes abundantly induced in nontransformed clones after 3 days of RA treatment, are expressed in N-*ras*-transformed cells only after 10 days of RA treatment. At this time, the cells' growth is arrested at very high density, and no differentiated morphologic characteristics are observed. Constitutive expression of a transfected Hox 1.4 gene under the control of a simian virus 40 promotor leads to differentiated cell morphology similar to that of the RA-induced phenotype and restores the growth-inhibitory effects of RA in N-*ras*-transformed cells. These observations provide evidence that enhanced proliferation in N-*ras*-transformed cells compromises teratocarcinoma cell differentiation by a mechanism that transiently suppresses homeobox gene induction and implies a central role for homeobox genes in RA-induced cell differentiation. We conclude that stimulation of a putative growth factor signal pathway, associated with *ras*-induced proliferation, transiently suppresses the induction of transcription factors functionally involved in cell growth and differentiation.

There is a clear connection between cell differentiation and growth control. During development as well as during terminal cell differentiation, when cells cease to proliferate, growth is arrested and a highly differentiated phenotype appears. Even in tumor cells, in which growth control is somehow compromised, the degree of differentiation is generally inversely correlated with the rate of proliferation, so that the grade of differentiation has become an important parameter in the histologic evaluation of tumors.

Cell growth and differentiation both involve the interaction of environmental signal molecules, transduction of these signals to the cell nucleus, and a cascade of regulated gene expression. At some level, convergence of these signal pathways seems to lead to finely tuned growth-regulatory and differentiation-specific gene expression. Retinoic acid (RA) has been identified as a key morphogen in vertebrate development (9, 41, 45) and as a potent regulator of both adult and embryonic cell differentiation (22, 35). Three different human nuclear RA receptors (RARs), alpha RAR (22, 34), beta RAR (8), and gamma RAR (25), have been cloned and found to be members of the steroid receptor family (18). However, the genes involved in further transducing the RA signal are largely unknown.

Homeobox genes are a group of transcription factors (15, 17, 23, 30) which in fruit flies play an important role in this signal transduction toward phenotype-specific gene expression. Specific activation of homeobox genes during RA-induced differentiation has been reported in a variety of teratocarcinoma cell lines (13, 16, 26); the activation requires RA, which cannot be replaced by any other differentiation-inducing agent (14). Vertebrate homeobox genes (47) are located in groups on four different genomic clusters (HOX 1, 2, 3, and 4) on chromosomes 7, 17, 12, and 2, respectively (reviewed in reference 37). Homeobox gene activation in vivo is tightly controlled in specific temporal and spatial patterns, which reflect the location of individual homeobox genes in the genomic cluster in the *Drosophila* and mouse genomes (1). Recent work with N-Tera-2 cells shows that the nine genes on cluster HOX 2 are sequentially activated starting from the cluster's 3′ end gene Hox 2.9 (36). Since treatment with cycloheximide destroys this temporal gene expression control, the conclusion has been that it is achieved by proteins with relatively short half-lives.

The molecular mechanisms by which *ras* influences gene expression are less well understood because the intermediate steps between the *ras* signal at the cell membrane and the nuclear response remain to be elucidated (4). In many cell systems, however, *ras* activation stimulates cell proliferation and triggers DNA synthesis by a pathway held in common with growth factor receptors (10). *ras* has also been shown to cause specific alterations of gene expression (33). In a variety of cell systems, expression of a *ras* oncogene results in morphologic transformation (e.g., in NIH 3T3 cells and rat embryo fibroblasts), whereas in PC-12 pheochromocytoma cells (5) and embryonic neurons (6), a highly differentiated neural phenotype can be induced by *ras* oncopro-

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teins. This phenotype is indistinguishable from that induced by nerve growth factor, which provides further evidence that *ras* can ultimately cause differentiation-specific changes in gene expression.

Teratocarcinoma cells are an in vitro system for studying early embryonic development (27), and they are also useful for studying the differentiation of proliferating stem cells into phenotypes characteristic of mature tissues (for a review of human cell systems, see reference 2). We used a series of cell clones from PA-1, a human cell line established from an ovarian teratocarcinoma (48), to study the effect of transformation by an activated N-*ras* oncogene on the expression of transcription factors involved in RA-induced cell differentiation. While these cells do not represent stem cells in culture, they can form embryoid bodies and respond to RA and hexamethylene *bis*-acetamide (HMBA). Since we have been able to express genes from a simian virus 40 (SV40) promoter (i.e., pSV2-neo), we believe that these cells possess some differ iated character. However, like murine teratocarcinoma c ls, PA-1 cells are permissive for infection by a retrovirus, gibbon ape leukemia virus, only after RA treatment (37a). Tumors derived from different subclones of PA-1 cells have a variety of histologies, indicating that these cells retain a degree of multipotency (39).

We show that cluster HOX 1 homeobox gene induction by RA follows a temporal pattern similar to that of cluster HOX 2 genes, whereas two independently derived N-*ras*-transformed cell clones that are less responsive to RA induced HOX 1 genes with severely delayed kinetics. The loss of function caused by this compromised homeobox gene induction can be reversed in part by constitutive expression of Hox 1.4 driven by an SV40 promotor.

MATERIALS AND METHODS

History of cell lines. PA-1 human teratocarcinoma cells were derived from a female ovarian germ cell tumor (48). S blines clone 6 and clone 1 were established by cloning PA-1 cells at limiting dilution from passages 40 and 63, respectively. Both clone 6 and clone 1 cells are nontumorigenic upon injection into nude mice. Whereas the clone 1 cells used in this study (passage 50 after cloning) become tumorigenic after expression of a single-copy *ras* or *myc* oncogene, the clone 6 cells used (passage 20 after cloning) remain resistant to *ras* transformation (for a review of all PA-1 sublines used in this study, see reference 39).

Subline 9113 is a spontaneously transformed cell clone, derived by injecting polyclonal PA-1 cells at passage 150 into nude mice and reestablishing these tumor cells into tissue culture. 9113 cells contain a G to A mutation at codon 12 in the N-*ras* gene. The causal role of this N-*ras* activation for tumorigenicity was proved by cloning the mutated N-*ras* gene under its own genomic promotor and then transfecting it into clone 1 cells. The frequency, short latency (6 weeks), and histologic appearance (neuroblastoma) of tumors derived from clone 1 N-*ras*-transformed cell clones are identical to these features in 9113 cells (38, 40). As a control, all pSV2-neo-transfected clone 1 cells are nontumorigenic. For the studies reported here, we chose to work with the subclone 6928, which has been recloned from a clone 1 N-*ras*-derived neuroblastoma. Extensive further characterization revealed that 6928 cells carry a single-copy plasmid with the mutated N-*ras* gene (40), express p21 protein levels equivalent to those of clone 1 and all other PA-1 sublines (11), exhibit no apparent cytogenetic alteration from clone 1 cells (40), and express the same isoenzyme pattern as all other PA-1 cells (data not shown). We chose clone 6928 from a group of clone 1 transfectants because it differs from clone 1 by only a single mutated N-*ras* gene and seems to represent a gene transfer-derived analog of the spontaneously mutated 9113 cells.

9117 cells are a spontaneously transformed subline derived from clone 6 cells carried for another 48 passages in vitro, after which they were injected into nude mice and reestablished in tissue culture. In contrast to PA-1 tumor cell clones that harbor a *ras* mutation, this non-*ras*-transformed tumor line, 9117, forms reproducibly mixed adenosquamous carcinomas at a lower frequency (30% of inoculated mice) and with a longer latency (12 to 14 weeks) than *ras*-transformed 6928 and 9113 cells.

Cell culture. All cells were cultured in modified Eagle's medium (MEM) supplemented with 7.5% fetal bovine serum. For differentiation, cells were seeded at 5×10^5 per 100-mm dish or at an equivalent cell density. The next day, the medium was replaced by fresh medium containing 10^{-5} M RA, and incubation was continued for 8 to 240 h. To obtain growth curves, 10^5 cells were seeded in 60-mm dishes and treated with RA for 1 to 10 days, and then the number of cells was counted with a Coulter counter (Coulter, Hialeah, Fla.).

Cell transfections. Eight million exponentially growing cells were resuspended in 400 μl of H-buffer (20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid]-buffered saline, pH 7.2) and added to 400 μl of H-buffer complemented with each DNA. Transfections were performed with a Bio-Rad electroporator at 280 V and 500 μF. Cells were kept on ice for 10 min, electroporated, kept for 10 min on ice again, and finally distributed into four 100-mm dishes. Selection was started 48 h later with G418 (200 μg/ml; GIBCO, Grand Island, N.Y.) or hygromycin (100 μl/ml; CalBiochem, San Diego, Calif.).

Isolation of RNA and Northern (RNA) blot analysis. Total cellular RNA was isolated by guanidine isothiocyanate lysis and centrifugation through a cesium chloride gradient (12). Polyadenylated [poly(A)$^+$] RNA was selected twice by oligo(dT) spin columns (Pharmacia LKB, Piscataway, N.J.); 1.25 or 2.5 μg of RNA was loaded per lane on 1.2% agarose–formaldehyde gels and blotted onto nylon membranes (Schleicher & Schuell, Keene, N.H.). Double-stranded probes were labeled to specific activities between 1×10^9 and 3×10^9 cpm/μg with a random primer kit (Bio-Rad, Richmond, Calif.). Hybridizations were performed in 45% formamide–5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–1% sodium dodecyl sulfate (SDS)–100 μg of salmon sperm DNA per ml–50 μg of tRNA per ml for 16 h at 42°C. Final washes were done in 0.2× SSC–0.01% SDS at 55°C. Hox 1.4 and Hox 1.3 cDNA fragments generated by polymerase chain reaction (PCR), and partial genomic clones (provided by E. Boncinelli) of Hox 1.6, Hox 1.11, Hox 1.5, and Hox 1.2 were used as probes.

Isolation of recombinant cDNA clones. We used 2.5 μg of twice-poly(A)$^+$-selected RNA to construct a cDNA library from clone 1 cells treated for 120 h with RA. Synthesis of cDNA and addition of linkers were performed with an oligo(dT)-primed cDNA synthesis kit (Pharmacia) according to the manufacturer's instructions. Ten percent of the cDNA was ligated into *Eco*RI-cut and dephosphorylated lambda ZAP II (Stratagene, La Jolla, Calif.) and packaged with Gigapack Gold extracts (Stratagene), resulting in 11.8×10^6 plaques. Screening and in vivo excision of plasmids from

positive phages were done according to the manufacturer's instructions.

PCR. For first-strand cDNA synthesis, 10 μg of total RNA was pelleted in an Eppendorf tube, washed, dried, redissolved in 13.5 μl of water, and heated for 5 min at 65°C. Two microliters of 10× buffer (0.5 M Tris [pH 8.5], 0.4 M KCl, 0.1 M $MgCl_2$, 4 mM dithiothreitol [DTT]), 2 μl of dATP, dTTP, dGTP, and dCTP (10 mM each), 1 μl of oligo(dT) primer (1 μg/μl) with a *Sal*I recognition sequence at the 5′ side, 0.5 μl of RNasin (Pharmacia), and 1 μl of avian myeloblastosis virus reverse transcriptase (30 U; Boehringer-Mannheim, Germany) were added and incubated at 43°C for 1 h. For amplification, the buffer was adjusted with 10 μl of 450 mM KCl, 0.1% gelatin, and 1 μl of a homeodomain-specific oligonucleotide (5′-TGGATCC-ATCAAGATTTGGTTCCA GAACCG-3′, 1 μg/μl [the first seven nucleotides are the added *Bam*HI site]), brought to a final volume of 100 μl, covered with 50 μl of mineral oil, and denatured for 3 min at 95°C. After 2.5 U of *Taq* polymerase were added, 30 cycles of amplification were performed. Annealing was done for 2 min at various temperatures (37, 42, or 55°C), extension was done for 5 min at 63°C or 3 min at 73°C, and denaturing was done for 1 min at 95°C. After 29 cycles, an extra unit of enzyme and 2 μl of ^{32}P-labeled dCTP were added, and a final extension was performed for 10 min at 73°C. The reaction mixture was finally extracted once with phenol-chloroform and then precipitated with ethanol. For analysis, 5 to 10% of the product was separated by polyacrylamide gel electrophoresis and exposed overnight on X-ray film (XAR-5; Eastman Kodak, Rochester, N.Y.). For subcloning purposes, 50% of the remaining mixture was cut with *Bam*HI and *Sal*I, separated by electrophoresis, and exposed wet on X-ray film at room temperature. Individual bands were sliced from the gel, extracted, and ligated into the sequencing vector pGEM-3Z (Promega, Madison, Wis.).

RESULTS

Transformation by N-*ras* inhibits RA-induced growth arrest and morphological differentiation. To determine the effect of an activated N-*ras* oncogene on RA-induced growth arrest and cell differentiation, we studied five different subclones from PA-1 cells that vary in their response to RA: two nontumorigenic clones (clone 6 and clone 1 cells), two N-*ras*-transformed tumorigenic clones (6928 and 9113 cells), and one non-*ras*-transformed tumorigenic clone (9117 cells). The history and main features of these sublines are briefly summarized under Materials and Methods and have been described in detail before (11, 38–40).

During a 5-day treatment with 10^{-5} M RA, the growth of clone 6 and clone 1 cells arrested at 20% of the cell density of untreated cultures, and a flat, epitheliallike phenotype (Fig. 1) developed that could not be induced in the highly tumorigenic, N-*ras*-transformed 6928 and 9113 cells. RA-treated cultures of the transformed 6928 and 9113 cells reached 60 and 80%, respectively, of the cell density of untreated cells and retained their undifferentiated morphologic characteristics (Fig. 1). In contrast, the non-*ras*-transformed cell clone 9117 was similar to the nontumorigenic clones in its responsiveness to RA. Both nontumorigenic clones, clone 6 and clone 1 cells, exhibited an RA-induced differentiated cell morphology and negative regulation of proliferation (Fig. 1). While 9117 cells responded to RA, they are tumorigenic and not contact inhibited. Untreated 9117 cells, like 9113 cells, reached about twice the cell number of untreated clone 6 and clone 1 cells in 10 days. Repeated attempts to transfect 9117 cell DNA into NIH 3T3 cells did not reveal the presence of an activated *ras* or any other oncogene. Interestingly, 9117 cells reproducibly form adenosquamous carcinomas in nude mice (39), which raises the possibility that the N-*ras* oncogene in 6928 and 9113 cells shifts in vivo differentiation toward a neurogenic lineage. Unfortunately, we have not been able to identify the induction of any neural antigen by RA in the N-*ras*-transformed cell lines in vitro. 9117 cells, like other PA-1 cell lines, downregulate the expression of the embryonal surface antigen Tra 1–60 (3) after 5 days of RA treatment (2a).

These results demonstrate that N-*ras* transformation of PA-1 cells compromises the effects of RA on cell differentiation and growth regulation in vitro. A second, still unidentified mechanism leads to tumorigenic transformation of 9117 cells but does not change the morphologic or growth features of the RA response.

Isolation of homeobox gene cDNA fragments by PCR. We analyzed homeobox gene expression in PA-1 cells, intending to use them as molecular markers for RA-induced cell differentiation and to examine whether their expression was altered in N-*ras*-transformed cell lines. To identify such genes rapidly, we did PCR with a homeodomain-specific oligonucleotide and an oligo(dT) nucleotide to amplify first-strand cDNA templates. To facilitate the subcloning of reaction products, both primers had 5′ restriction sites. PCR was performed with cDNA templates from untreated clone 1 and 6928 cells and from cells treated for 120 h with RA.

PCR products that appeared to be differentially expressed, based on analysis of PCR products by gel electrophoresis, were subcloned. Two such fragments could be identified by sequencing as fragments of the human Hox 1.4 and Hox 1.3 cDNAs.

The PCR-amplified fragment of Hox 1.3 contains an internal *Bam*HI site. Because we cut the PCR products for subcloning with *Bam*HI and *Sal*I, we cloned and sequenced only the 3′ half of the amplified cDNA fragment between nucleotide 1810 and the adenine-rich area starting at nucleotide 2056 (42). The sequence of this fragment is identical to the previously determined human Hox 1.3 sequence, and it was used as a probe for Northern blot analysis. This probe does not contain homeodomain sequences, does not cross-hybridize with Hox 1.4 plasmids, and therefore represents a small but specific probe for Hox 1.3.

Isolation of complete Hox 1.4 cDNA. We constructed a cDNA library from clone 1 cells treated with RA for 120 h and screened it with the PCR-derived fragment to obtain a human full-length cDNA of Hox 1.4. We isolated 31 independent cDNA clones from 250,000 lambda ZAP II phages and sequenced three overlapping cDNA clones fully on both strands. The nucleic acid sequence and the predicted peptide of the longest open reading frame are shown in Fig. 2B. As indicated in Fig. 2A, Hox 1.4 contains two very conserved protein regions. Of the 24 N-terminal amino acids, 24 are identical among four related Hox genes (Hox 1.4, Hox 2.6, Hox cp19, and Hox c13) and the first 22 are identical between mice and humans. The homeodomain with an additional C-terminal 114 amino acids has 9 amino acids that are different, with three of the changes being conservative between mice and humans (21). Both domains are also highly conserved within this subgroup of human homeobox genes related to Hox 1.4, but less so than between mice and humans (37). Of the N-terminal 206 amino acids, there are 45 changes between mice and humans (21). The predicted molecular mass of the 320-amino-acid Hox 1.4 protein is about 34 kDa, with an estimated pI of 10 because of an

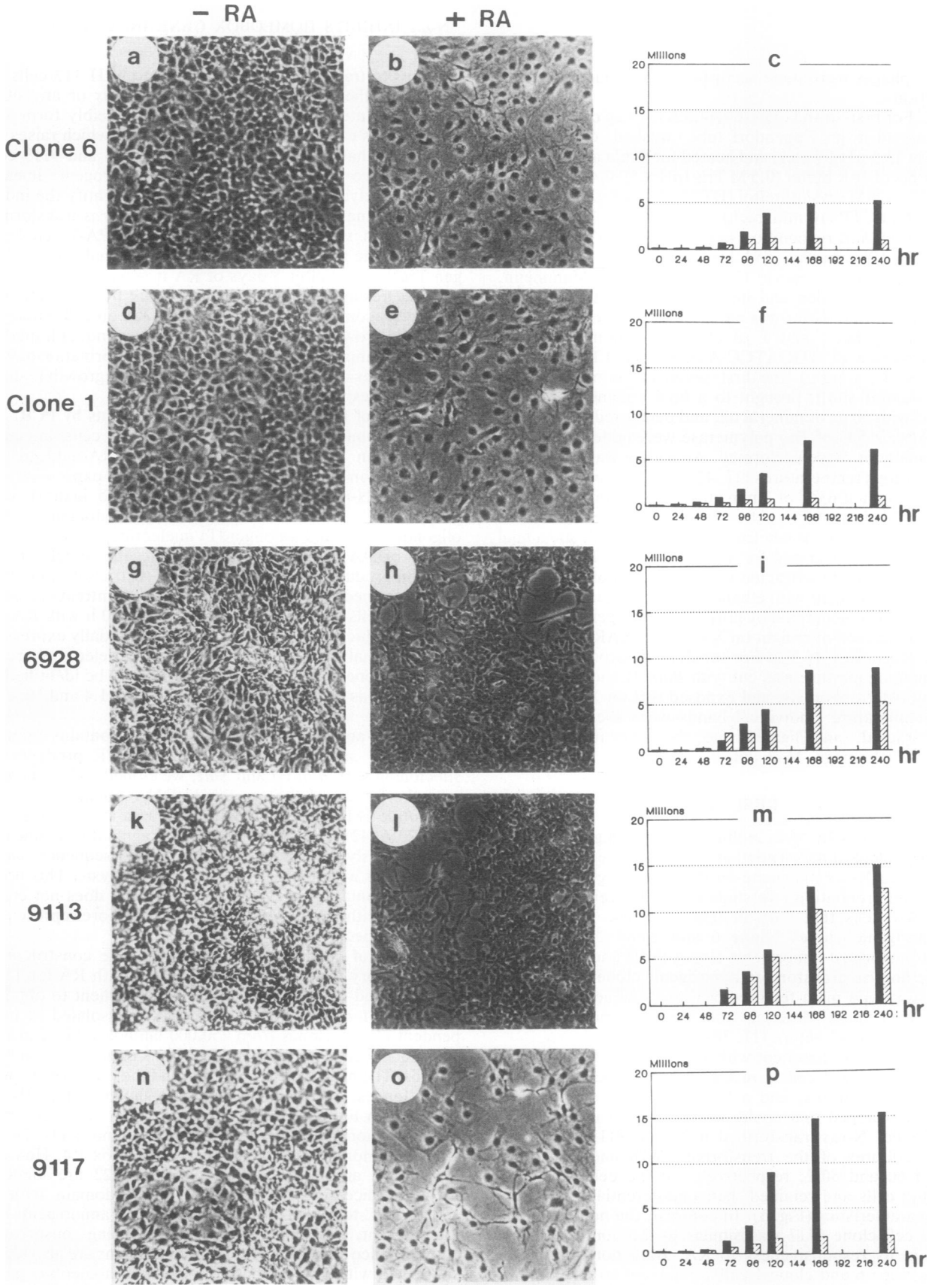


FIG. 1. Parameters of growth regulation and morphology in response to RA. Five PA-1 cell sublines, clone 6 (a, b, c), clone 1 (d, e, f), 6928 cells (g, h, i), 9113 cells (k, l, m), and 9117 cells (n, o, p), were tested. Panels on the left show untreated cells exhibiting undifferentiated, embryonal cell morphology. Panels in the center display cells after 5 days of treatment with 10^{-5} M RA. Magnification, ×63. Growth curves for cells grown in the absence (solid bars) or presence (hatched bars) of RA are shown at the right.

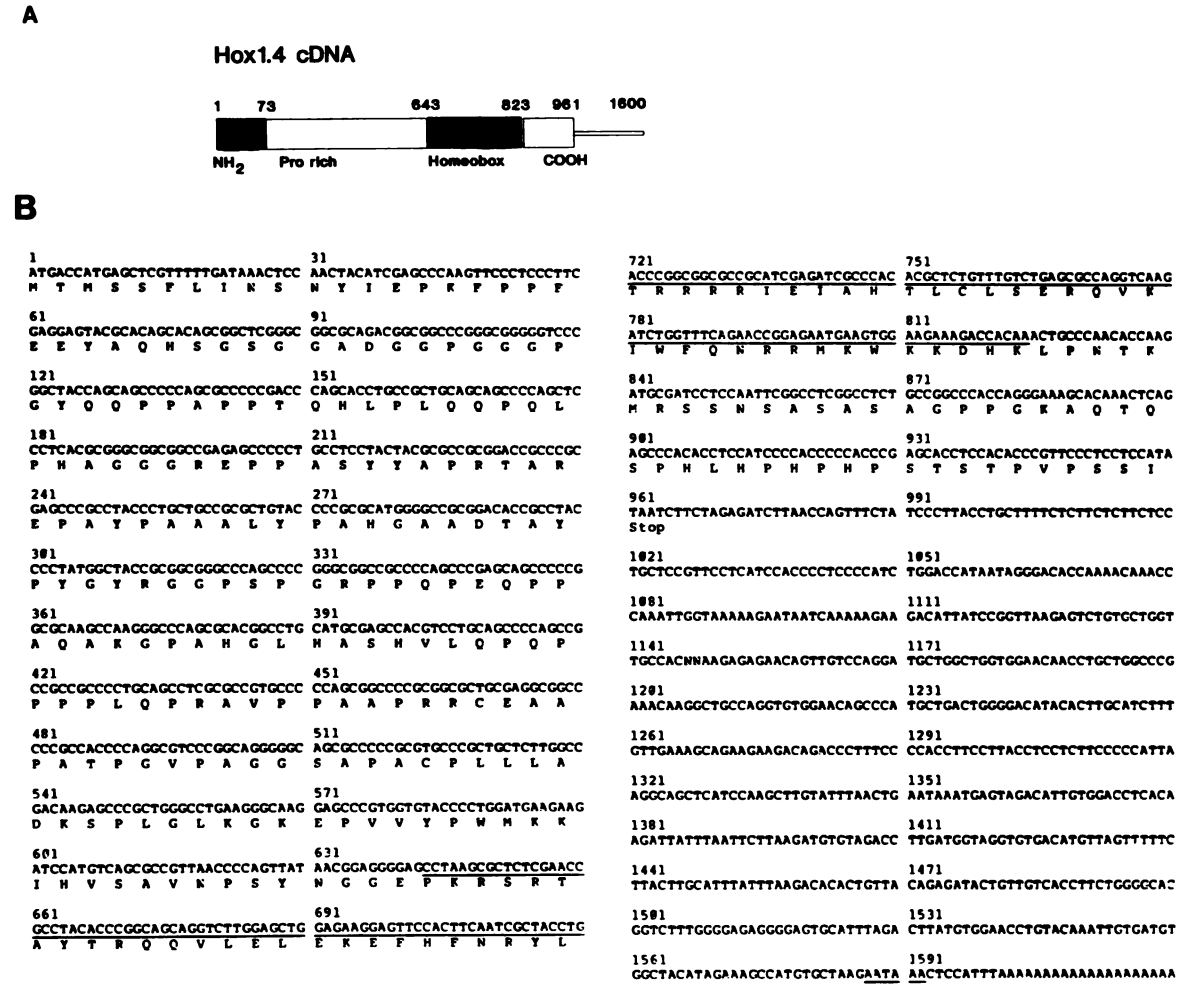


FIG. 2. DNA and amino acid sequence of human Hox 1.4. (A) Schematic representation of the full-length cDNA. The translated area (nucleotides 1 to 960) is enlarged; solid boxes represent conserved protein domains. The proline-rich area (32 prolines in 120 amino acids) from nucleotides 94 to 454 is indicated. (B) Nucleotide sequence and predicted amino acids of human Hox 1.4. The homeobox and polyadenylation signal are underlined.

excess of basic amino acid residues (5% lysines, 6% arginines, and 4.7% histidines versus only 1.3% aspartates and 4.4% glutamates).

Possibly an important feature of the region between the N-terminal domain and the homeodomain is the extremely high content of prolines (26.5% of all amino acid residues in the region from nucleotides 94 to 454). Such a proline-rich domain has been identified in other transcription factors, i.e., AP-2 (46) and Oct-3 (31), and has been shown in CTF/NF-1 to be indispensable for transactivator function (29).

Cluster HOX 1 homeobox genes are activated by RA in PA-1 cells and altered in expression by N-*ras* transformation. We used PCR-derived fragments from Hox 1.4 and Hox 1.3 and four genomic clones from Hox 1.6, Hox 1.11, Hox 1.5, and Hox 1.2 as probes for Northern blot analysis to determine HOX 1 gene expression during RA-induced differentiation of PA-1 cells, analyzing the induction time course in mRNA from clone 1 cells treated for 0, 8, 24, 48, 72, 96, 120, and 240 h with 10^{-5} M RA. The Northern blots shown in Fig. 3A revealed that HOX 1 homeobox genes were induced beginning at the 3′ end of the cluster. Hox 1.6, similarly identified from F9 murine teratocarcinoma cells as ERA-1 (26), was induced by 4 h of RA treatment, 1.11 partially at 8 h and fully after 24 h, 1.5 at 24 h, 1.3 and 1.4 between 48 and 72 h, and 1.2 at 96 h (Fig. 3A and B). The mRNA sizes are: Hox 1.6, 2.4 and 2.6 kb; Hox 1.11, 1.2 and 1.6 kb; Hox 1.5, 3.5 kb; Hox 1.4, 1.8 kb; Hox 1.3, 1.9 kb; and Hox 1.2, 2 kb. To confirm the induction of these homeobox genes in a second nontumorigenic, highly RA-responsive PA-1 clone, we analyzed the mRNA of clone 6 cells for the presence of HOX 1 gene mRNA. None of the six HOX 1 genes was expressed in the absence of RA, but all were detected on Northern blots after 5 days of RA treatment (Fig. 4A).

To study HOX 1 gene expression in N-*ras*-transformed PA-1 cells, we analyzed mRNA from 6928 cells (N-*ras*-transfected clone 1 cells) and 9113 cells (spontaneous N-*ras* mutant) at selected time points during treatment with 10^{-5} M RA. As shown in Fig. 4A and B, the genes located at the 3′ end of the cluster HOX 1, Hox 1.6 and Hox 1.11, which were induced within 8 h and expressed after 5 days of treatment in clone 1 cells, were unchanged (Hox 1.6) or slightly reduced (Hox 1.11) in level in the *ras*-transformed cells. In contrast, four other HOX 1 homeobox genes (Hox 1.5, 1.4, 1.3, and

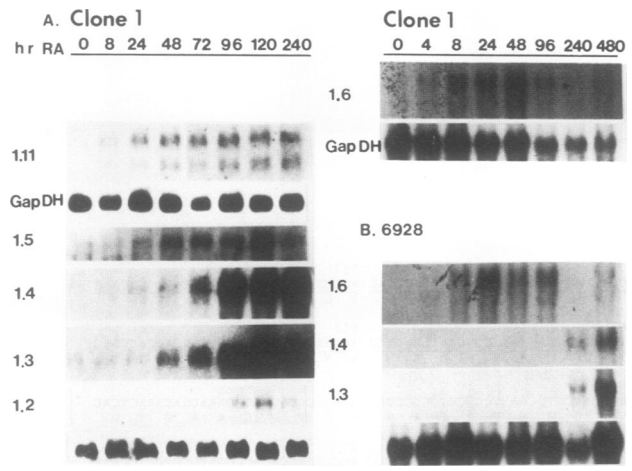


FIG. 3. Induction time course of HOX 1 homeobox genes after treatment with 10^{-5} M RA in clone 1 (A) and 6928 (B) PA-1 cells. (A) Five micrograms of singly poly(A)$^+$-selected RNA from clone 1 cells treated for 0, 8, 24, 48, 72, 96, and 120 h was loaded per lane, Northern blotted, and hybridized with partial cDNA probes for Hox 1.3 and Hox 1.4 or partial genomic probes for Hox 1.6, Hox 1.11, Hox 1.5, and Hox 1.2 (indicated on left). Ten micrograms of total RNA was analyzed for Hox 1.6 mRNA (Northern blot shown on top of right panel). (B) Ten micrograms of total RNA from 6928 cells treated for 0, 4, 8, 24, 48, 96, 240, and 480 h were analyzed for Hox 1.6, Hox 1.4, and Hox 1.3 mRNA. To control RNA loading, all blots were rehybridized with a rat cDNA probe for glyceraldehyde-3-phosphate dehydrogenase (GapDH).

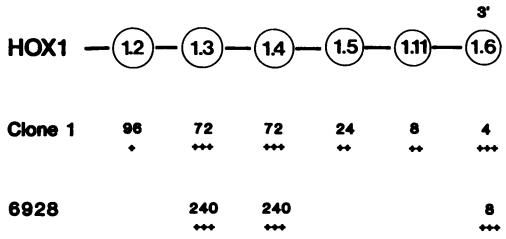


FIG. 5. Time course of HOX 1 homeobox gene induction in nontransformed (clone 1) and N-*ras*-transformed (6928) PA-1 teratocarcinoma cells during treatment with RA. The location of HOX 1 genes on the genomic cluster is indicated on top (from 5′ to 3′). The time (in hours) during RA treatment at which these genes are first expressed in clone 1 and 6928 cells is indicated. The level of expression was evaluated semiquantitatively: signal on Northern blot detected in twice poly(A)$^+$-selected RNA after 3 days (+) or 24 h (++) of exposure and signal on Northern blot detected in unselected RNA after 48 h of exposure.

1.2) failed to be induced during the first 5 days of treatment in the *ras*-transformed cells (Fig. 4).

We then analyzed the time course of induction of the three most abundant HOX 1 genes (Hox 1.6, 1.4, and 1.3) in 6928 cells in further detail. As the Northern blot data in Fig. 3B show, activation of these three HOX 1 genes was not entirely suppressed in 6928 cells but was significantly delayed. Hox 1.6 was first detected after 8 rather than 4 h of treatment, as in clone 1 cells, and Hox 1.4 and Hox 1.3 were partially induced after 10 days and fully induced at 20 days of RA treatment rather than within 2 to 3 days (Fig. 3B). Figure 5 summarizes induction time course data for clone 1 and 6928 cells and illustrates the relationship between temporal pattern of expression and the physical location of the HOX 1 genes in the genomic cluster.

In addition, we analyzed mRNA from the non-*ras*-transformed 9117 cells, which are highly responsive to RA, at selected time points of RA treatment (Fig. 4B). We found that the temporal pattern of HOX 1 gene induction in these cells is apparently undelayed compared with induction in clone 6 and clone 1 cells. Thus, the mechanism by which 9117 cells became tumorigenic does not seem to modify the kinetics of HOX 1 gene induction.

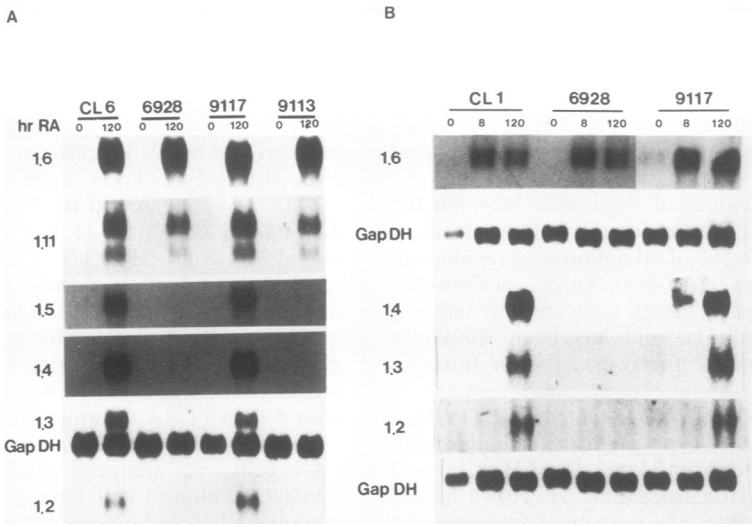


FIG. 4. HOX 1 homeobox gene expression after 120 h of treatment with 10^{-5} M RA in clone 6, clone 1, 6928, 9113, and 9117. (A) Doubly poly(A)$^+$-selected RNA (1.25 μg) from clone 6 (CL6), 6928, 9117, and 9113 cells, untreated or treated for 120 h with 10^{-5} M RA, was hybridized with the HOX 1 homeobox probes indicated on the left. Estimated sizes of mRNA are: Hox 1.6, ca. 2.2 and 2.4 kb; Hox 1.11, ca. 1.8 and 1.4 kb; Hox 1.5, ca. 2.7 kb; Hox 1.4, ca. 1.8 kb; Hox 1.3, ca. 1.85 kb; and Hox 1.2, 1.8 kb. (B) Doubly poly(A)$^+$-selected RNA (1.25 μg) from clone 1 (CL1), 6928, and 9117 cells, untreated or treated for 8 or 120 h with 10^{-5} M RA, was hybridized with the HOX 1 homeobox probes indicated on the left.

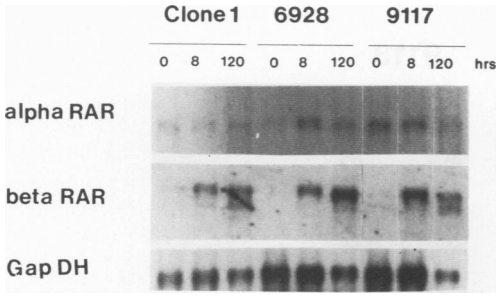


FIG. 6. RAR expression in response to RA in clone 1, 6928, and 9117 PA-1 teratocarcinoma cells. Doubly poly(A)$^+$-selected RNA (1.25 μg) from cells treated for 0, 8, and 120 h with 10^{-5} M RA (indicated on top) was loaded per lane. The probes were alpha RAR human cDNA (gift from R. Evans), beta RAR human cDNA (gift from P. Chambon), and GapDH.

Expression of RA receptors. To study whether differing responses to RA and the differing ability to induce homeobox genes in clone 1 and 6928 cells result from alterations in RAR expression, we used Northern blot analysis to measure mRNA levels of alpha, beta, and gamma RAR. Summarized in Fig. 6, our data showed a constitutive low level of a single alpha RAR mRNA (ca. 2.7 kb). Beta RAR was not expressed in untreated PA-1 cells; an mRNA of about 3 kb was induced within 8 h of RA treatment, and a second slightly smaller message appeared after 5 days. That this band is not the gamma RAR was shown by our inability to observe any hybridization when we used gamma RAR cDNA as a probe (data not shown). Thus, an identical pattern of RAR expression appeared in the three sublines, unaffected by N-*ras* transformation in 6928 cells.

Constitutive overexpression of Hox 1.4 in PA-1 cells confers RA treatment effects. The considerable delay of HOX 1 homeobox gene induction in the N-*ras*-transformed cell lines means that most of these gene products are absent between days 1 and 5 of RA treatment, when inhibition of growth is accomplished by some still-unknown RA-induced gene-regulatory mechanism. We therefore tested whether the absence of expression of several HOX 1 genes in the N-*ras*-transformed cell lines is causally linked to their reduced responsiveness to RA. To study this issue, we stably transfected the complete Hox 1.4 cDNA into clone 1, 6928, and 9113 cells and analyzed whether constitutive expression of this gene had any effects similar to RA treatment on cell morphology or growth inhibition.

Our experimental strategy was to insert the Hox 1.4 cDNA into the SV40 expression vector pSG5 (Stratagene), excise the expression cassette containing the SV40 promoter, Hox 1.4 cDNA, and polyadenylation signal, and transfer it into the *Eco*RI site of pSV2-neo or the *Ban*II site of pSV2-hygro. Two of eight neomycin-resistant clones from clone 1 cells, one of five neomycin-resistant clones from 9113 cells, and 2 of 12 hygromycin-resistant clones from 6928 cells were shown by Northern blot analysis to express Hox 1.4 cDNA in the absence of RA treatment (Fig. 7A). Interestingly, even without RA treatment, these Hox 1.4 expressor clones resembled untransfected or *neo*-transfected clone 1 cells after RA treatment (Fig. 7B). Flat epitheliumlike cells as well as cells with long spindly extensions were observed. These phenotypes became even more abundant during treatment with 10^{-5} M RA, and few undifferentiated cells were left.

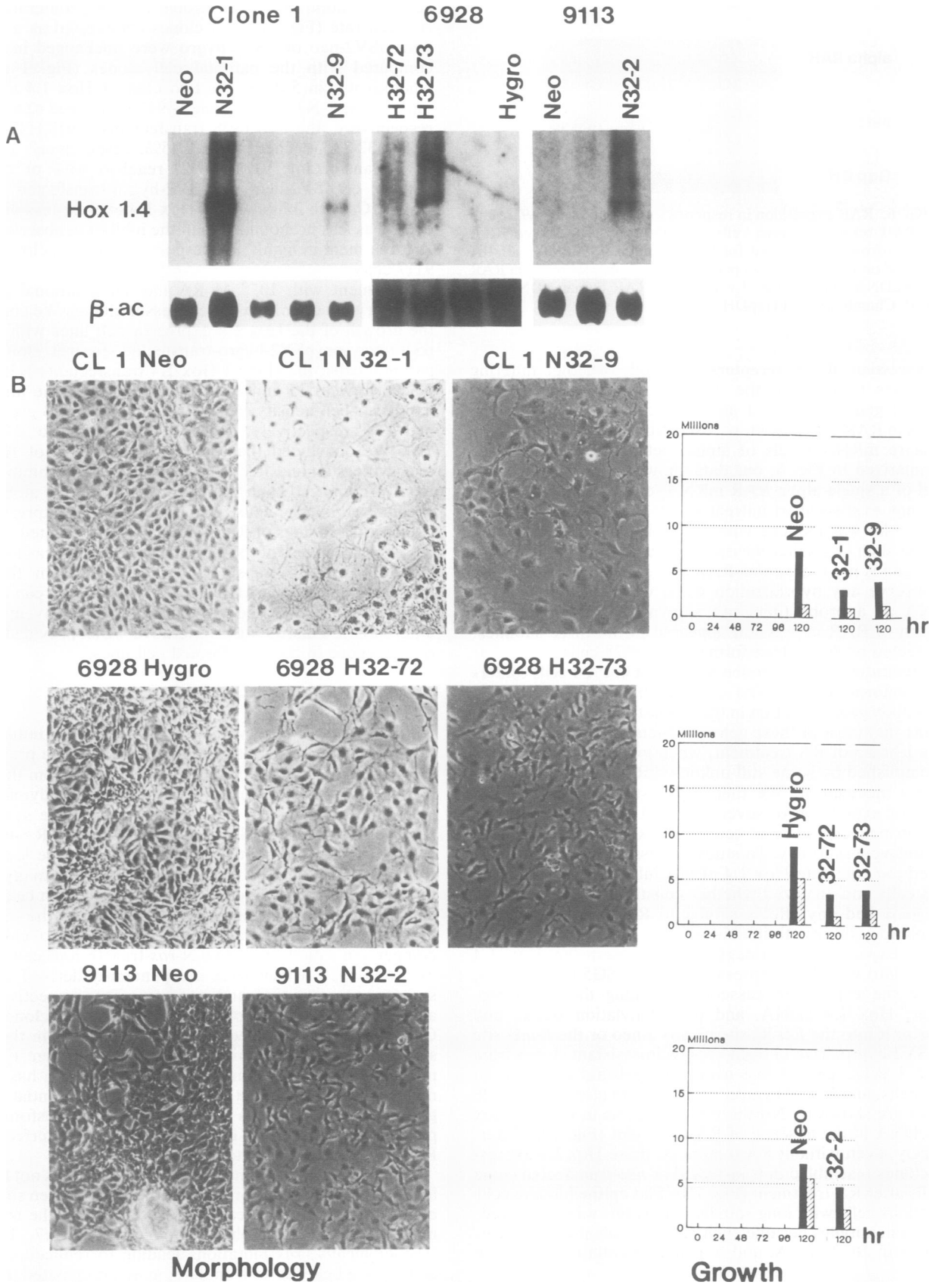
Measuring the growth characteristics of Hox 1.4 transfectants, we found that both *ras*-transformed 6928 and 9113 cells and nontransformed clone 1 cells proliferated at a reduced rate (Fig. 7B). All clones transfected as a control with pSV2-neo or pSV2-hygro were unchanged in growth compared with the parental cell clones (Fig. 1 and 7), whereas within 5 days, the two clone 1 Hox 1.4 transfectants, clone 1-N32-1 and clone 1-N32-9, reached 42 and 54%, respectively, the two 6928 transfectants, 6928-H32-72 and 6928-H32-73, reached 40 and 45%, respectively, and one 9113 transfectant, 9113-N32-2, reached 65% of the cell number of pSV2-neo- or pSV2-hygro-transfected control clones. Growth inhibition by Hox 1.4 overexpression, however, was less pronounced than the inhibition observed after RA treatment in highly RA-responsive clone 1, clone 6, and 9117 cells.

Treatment with 10^{-5} M RA had an additional growth-inhibitory effect on Hox 1.4 expressor clones. We compared the growth of the Hox 1.4 expressor cell lines with that of pSV2-neo or pSV2-hygro-transfected control clones and parental cells; the clone 1 Hox 1.4 transfectants N32-1 and N32-9 grew to 15 and 18%, respectively, of the untreated expressor cell density, 6928-H32-72 and H32-73 grew to 12 and 20%, respectively, and 9113-N32-2 grew to 31% (Fig. 7B). We concluded that the combined effect of Hox 1.4 overexpression and RA treatment on growth inhibition was more pronounced than the effect of either one alone. The absolute number of cells reached by Hox 1.4 expressor cell clones after 10 days of RA treatment still correlated with the RA responsiveness of the parental cell lines: it was lowest in clone 1 Hox 1.4 transfectants and highest in the 9113 transfectant. It appears that Hox 1.4 expression can reverse the lack of RA-induced growth arrest, circumventing the differentiation-disruptive proliferative signal provided by the *ras* oncogene in the transformed cell lines.

DISCUSSION

PA-1 cells, a system with which to study the influence of tumorigenicity on cell differentiation in vitro. We previously (39) described a series of cell clones derived from the PA-1 cell line that provide an in vitro system for studying multistep carcinogenesis. In this study, we used the system to analyze the effects of N-*ras* transformation on RA-induced cell growth and differentiation. Clone 6 and clone 1, derived from early-passage PA-1 cells, respond like many other teratocarcinoma cell lines to RA treatment, with cell differentiation and growth inhibition. We analyzed the morphologic and growth characteristics of these two clones in comparison with those of two N-*ras*-transformed cell clones, 9113 and 6928, which were independently derived through spontaneous mutation and gene transfer, respectively. In addition, we studied a non-*ras*-transformed cell clone, 9117. Our data, as summarized in Fig. 1, show that in the PA-1 system, we can distinguish between two different types of malignant transformation. N-*ras* transformation has a pronounced inhibitory effect on RA-induced differentiation and growth arrest, whereas another type of transformation, present in 9117 cells, does not seem to interfere with RA-induced gene-regulatory effects.

In vivo, activating point mutations in *ras* genes not leading to gross changes in p21 protein synthesis have been shown to contribute to multistep carcinogenesis, i.e., in the transformation of colon epithelium into carcinomas (7, 19, 44). Physiologically, *ras* mutations leading to reduced GTPase activity are believed to stimulate, in an unregulated fashion, an internal growth factor signal transduction pathway (4).


Clone 1
6928
9113
Neo
N32-1
N32-9
H32-72
H32-73
Hygro
Neo
N32-2
A
Hox 1.4
β-ac
B
CL 1 Neo
CL 1N 32-1
CL 1 N32-9
Millions
20
15
10
5
0
Neo
32-1
32-9
0 24 48 72 96 120
120
120
hr
6928 Hygro
6928 H32-72
6928 H32-73
Millions
Hygro
32-72
32-73
hr
9113 Neo
9113 N32-2
Millions
Neo
32-2
hr
Morphology
Growth

The precise components involved therein and the molecular consequences of its stimulation are, however, still poorly understood. We believe that the transfer of a single mutated N-*ras* gene under its natural promotor, as in the generation of 6928 cells from clone 1 cells, represents a valuable in vitro model for examining the molecular consequences of *ras* transformation. Since the response to RA is compromised in 6928 and 9113 cells, we have studied the regulation of homeobox genes and RARs, transcription factors believed to be causally involved in transducing the RA signal.

HOX 1 genes are induced by RA in a characteristic temporal pattern which is altered by *ras* transformation. Analysis of the RA-induced activation of six different HOX 1 homeobox genes revealed a characteristic temporal pattern of induction similar to the one recently described for nine different HOX 2 homeobox genes (36). The genes located closest to the 3′ end of cluster HOX 1, Hox 1.6 and Hox 1.11, were induced within a few hours of RA treatment, and the time course seems to parallel the induction of Hox 2.9 and Hox 2.8 on the 3′ end of cluster HOX 2. Induction of Hox 1.5, upstream of Hox 1.11, was observed at about 24 h, and genes located farther upstream on cluster HOX 1 were activated successively. The only discrepancy was the appearance of the Hox 1.3 mRNA slightly before the Hox 1.4 mRNA. Not only was the temporal order of induction by RA maintained for homologous genes on the HOX 1 and HOX 2 clusters, the time points of induction tended to match closely. Since the kinetics of HOX 1 and HOX 2 gene activation were so similar, we believe that a common gene-regulatory mechanism controls the temporal pattern of induction of homeobox genes and that this will likely be demonstrated for clusters HOX 3 and HOX 4 as well. Simeone et al. (36), using simultaneous treatment with RA and cycloheximide, found that the mechanism of successive delay was protein synthesis dependent. We speculate either that proteins with a relatively short half-life are involved or that repressor factors are induced by RA with different affinities to individual homeobox gene promoters.

Of particular interest was the significant delay of HOX 1 gene induction in two different N-*ras*-transformed cell clones. The two earliest-appearing genes, Hox 1.6 and Hox 1.11, were the only HOX 1 genes we detected in the N-*ras*-transformed cell lines during the first 5 days of RA treatment. All genes located 5′ upstream from Hox 1.11 which we analyzed, Hox 1.5, 1.4, 1.3, and 1.2, were not expressed during the first 5 days of RA treatment. As illustrated by the growth curves, N-*ras*-transformed cells have grown within 5 days to high density, and therefore a potential effect of homeobox gene expression after day 5 on formation of a differentiated phenotype can no longer be observed. Possibly, the growth arrest induced by high cell density causes the N-*ras*-transformed cells to downregulate growth-stimulatory signals and thereby allow homeobox gene induction.

ras oncogenes have been shown to affect differentiation in various cell systems. We and others have shown that transfection of activated *ras* oncogenes blocks in vitro differentiation of myoblasts into myotubes (32). In the present study, we showed that transformation by an N-*ras* oncogene inhibits PA-1 teratocarcinoma cell differentiation in response to RA. The inhibition involves a mechanism that has not been elucidated but is apparently independent of RAR expression and suppresses a number of HOX 1 genes at a time of RA treatment crucial for growth regulation. Whether homeobox genes are directly suppressed by a *ras*-controlled pathway or whether the delay of homeobox gene expression is an indirect effect of *ras* transformation, we do not know as yet. To address this question more specifically, we have cloned 5′ upstream regulatory sequences from the human Hox 1.4 gene, which will allow us to cotransfect *ras* with a suitable Hox 1.4 reporter gene and study the precise nature of its effect on the Hox 1.4 promotor.

Homeobox genes are molecular effectors of the RA signal pathway. When we expressed Hox 1.4 constitutively under an SV40 promotor, we observed that this homeobox gene conferred the same features as RA treatment, including morphologic changes and growth inhibition. Even in the N-*ras*-transformed cell lines, the growth-inhibitory effect and a morphology resembling that of RA-treated nontumorigenic cells were observed. We conclude, therefore, that homeobox genes are involved in transducing the RA gene-regulatory signal. The *ras*-transformed cells expressing the Hox 1.4 gene grow somewhat faster, however, and to higher densities than do nontransformed Hox 1.4 expressor clones. Growth-stimulatory and growth-inhibitory signal pathways seem to converge dynamically, and even in the presence of high Hox 1.4 mRNA levels, some growth stimulation by the activated N-*ras* gene can be observed. Expression of Hox 1.4 in *ras*-transformed cells yields a cell which can respond normally to RA. This tuning of cell growth and cell differentiation by different signal pathways allows the plasticity which is required for the formation of complex three-dimensional cell systems.

In light of the obvious complexity of the program of differentiation occurring in these cultured cells, it was somewhat surprising that expression of a single Hox gene could so dramatically alter the properties of the differentiating clone 1 cells and even more so those of the differentiation-resistant, *ras*-transformed 6928 and 9113 cells. Our initial perspective of the role of Hox genes in differentiation involved complex and sequential changes in expression of multiple Hox genes. It is possible that the Hox 1.4 protein interacts with an essential regulator of differentiation or proliferation and that its overexpression sequesters this factor from interacting with multiple Hox gene products or other transcription factors. Indeed, Hox 1.4 is not induced early after RA treatment as is Hox 1.6, which could act independently and might have been a more likely candidate for a Hox gene which could induce a cascade of expression of other transcription factors, including Hox genes, which would then

FIG. 7. Morphology and growth parameters of PA-1 cells stably transfected with an SV40-Hox 1.4 expression vector. (A) Constitutive expression of Hox 1.4 mRNA was established by Northern blot analysis of neomycin-resistant clone 1 and 9113 transfectants and hygromycin-resistant 6928 cells. Drug-resistant cell clones were isolated by using cloning rings, and small-scale RNA preparations were performed with from 1×10^7 to 2×10^7 cells grown in the absence of RA. Ten micrograms of total RNA from each was electrophoresed on agarose gels, blotted, and hybridized to Hox 1.4 cDNA. Blank lanes correspond to drug-resistant cell clones which did not express the Hox 1.4 cDNA. β-ac, β-actin. (B) Morphology of pSV2-neo- and pSV2-hygro-transfected control clones (left) and Hox 1.4 expressor clones (center) in the absence of RA treatment. Cells (10^5) were seeded in 60-mm dishes and grown for 5 days, and cell numbers were counted in a Coulter counter. Magnification, ×63. Graphs on the right exhibit cell numbers of control pSV2-neo- and pSV2-hygro-transfected control clones or Hox 1.4 expressor clones grown for 5 days in the absence (solid bars) or presence (hatched bars) of 10^{-5} M RA.

initiate differentiation. This paradox will hopefully be resolved as we express other Hox genes in PA-1 cell variants and study their effect on growth, differentiation, and changes in gene expression. Notably, preliminary experiments have not revealed an increase in Hox 1.3 expression in the cell clones overexpressing Hox 1.4.

It is intriguing to draw an analogy to molecular mechanisms of muscle differentiation. Muscle-specific transcription factors, such as myoD1 and myogenin, have been found to cause transcriptional activation of muscle-specific gene expression (43). The induction of these transcription factors, however, requires reduction of growth factors, usually achieved by exposing myoblasts to low concentrations of serum (differentiation medium), in which the cells do not proliferate. Thus, the antagonism between growth-stimulatory signals and transcription factors involved in the regulation of differentiation seems to be a general theme.

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