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Abundant expression of homeobox genes in mouse embryonal carcinoma cells correlates with chemically induced differentiation

(pluripotent stem cells/differentiation *in vitro*/retinoic acid/gene expression/development)

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ABSTRACT **Mammalian homeobox-containing genes might play a role in embryonal pattern formation. In favor of this view is the recently reported expression of such genes during mouse embryogenesis [Manley, J. L. & Levine, M. S. (1985) *Cell* 43, 1–2]. The embryo-derived stem cells and in particular the pluripotent embryonal carcinoma (EC) cell lines are generally considered as a valid model of early mouse development. Homeobox-containing genes were shown to be expressed in differentiating EC cells. We have analyzed the expression of several of these genes in three EC cell lines triggered to differentiate by alternative treatments in the presence or in the absence of retinoic acid. In both types of conditions, C17S1 (clone 1003) and PCC7.S Aza R1 EC cells were induced to differentiate into mainly neurones, and PSA-1 EC cells were induced to differentiate into a large spectrum of tissue derivatives. Induction to high levels of expression of several homeobox-containing genes during differentiation occurs only in the presence of retinoic acid. Nonchemical treatment triggering differentiation does not lead to detectable expression of these genes. Accumulation to high amounts of homeobox-containing gene transcripts in these experiments seems to correlate with retinoic acid-induced EC cell differentiation rather than with EC cell differentiation as such.**

The homeobox is a 180-nucleotide-long protein-encoding DNA sequence discovered in several genes regulating development in *Drosophila*. Homeobox sequences present in the *Drosophila melanogaster* Antennapedia (*Antp*) homeotic gene and in the engrailed segmentation gene are prototypes of two classes of homology (1). The homeobox sequence has been conserved through evolution and is found in vertebrates, including mice and humans (2). Mammalian homeobox-containing gene transcripts have been detected in mouse and human embryos from gastrulation through organogenesis and later development (3–10). Embryo-derived mouse stem cell lines (EK or ES) (11, 12) and embryonal carcinoma (EC) cell lines (13) have been used as biological models for pre- and early postimplantation embryos. Because transcription analysis at very early stages of mouse embryogenesis would require a large number of embryos and because cell lines can be more easily submitted to culture manipulations than developing embryos, expression of homeobox-containing genes has been analyzed in differentiating EC and EK or ES cell lines.

The *Antp*-related *H24.1* mouse homeobox-containing gene, located on chromosome 11 (8), was reported to be expressed at an extremely low level after EK stem cells had been induced to differentiate by being grown as aggregates in the absence of feeder cells and subsequently plated onto a tissue culture surface (8). An independent isolate of *H24.1* did not allow any expression of this gene to be detected in PSA-1 EC cells similarly induced to differentiate (6). However, the human homolog of *H24.1*, *Hu1*, was shown to be strongly induced in retinoic acid-treated NT2/D1 human teratocarcinoma cells (6). The *Antp*-related *m6-12* mouse homeobox-containing gene (3), located on chromosome 6 (14), was shown to be induced during differentiation of F9 and P19 EC cells in the presence of retinoic acid (3) and during dimethyl sulfoxide-induced differentiation of P19 (15).

We followed the expression of these mouse homeobox-containing genes during EC cell differentiation induced either by a nonchemical treatment or by the same treatment in the presence of retinoic acid. We report that in three cell lines, a strong activation of these genes occurs only when retinoic acid is present during cell differentiation. Nonchemical treatment does not give rise to a detectable accumulation of homeobox transcripts.

MATERIALS AND METHODS

Cell Cultures. C17S1 (clone 1003) EC cells (C1003 cells) (16) were grown in a 1:1 mixture of Dulbecco and Vogt modified Eagle's/Ham's F12 medium (DF medium) supplemented with 7.5% fetal calf serum. Serum-free medium (17) was DF medium supplemented with insulin (5 μg/ml), transferrin (50 μg/ml), and selenium (25 nM) instead of serum. All-*trans*-retinoic acid (Sigma) was stored frozen at 10 mM in dimethyl sulfoxide. Retinoic acid concentration was 0.1 μM in serum-free medium instead of 1 μM in serum-containing medium due to the higher toxicity of retinoic acid in the absence of serum.

PCC7-S Aza R1 EC cells (PCC7 cells) (18) were grown in Dulbecco and Vogt modified Eagle's medium supplemented with 7.5% fetal calf serum. Differentiation protocols are described in the text: they were essentially according to ref. 19. PSA-1 NG2 EC cells (PSA-1 cells) (20) were grown on a layer of mitomycin C-treated mouse STO fibroblast feeder cells (21) in DF medium supplemented with 10% fetal calf serum. Differentiation into many cell types occurred after the cells were plated without feeder cells for 3 days (aggregate formation), grown as aggregates in bacterial Petri dishes for 3 days, and plated onto tissue culture dishes for various periods of time. Either retinoic acid (1 μM) was added for 2 days during aggregate formation and the cells were harvested thereafter or retinoic acid was present during 7 days from the moment the aggregates were grown in suspension until 4 days after plating. Control PSA-1 cultures were treated in the same way, but in the absence of retinoic acid.

RNA Isolation. Total cellular RNA was purified according to ref. 22. Poly(A)$^+$ RNA was separated by oligo(dT)-cellulose chromatography.

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Abbreviations: EC cells, embryonal carcinoma cells; EK and ES cells, embryonic stem cells; SSEA, stage-specific embryonic antigen.

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RNA Blot Analysis. Poly(A)$^+$ RNA (10 μg per lane) was fractionated by electrophoresis in 1.2% agarose slab gels containing formaldehyde (6.6%). RNA was transferred onto nitrocellulose filters (Schleicher & Schuell) and hybridized with nick-translated DNA probes (approximately 10^8 cpm/μg). Mouse homeobox DNA probes were H24.1 (8) and m6-12 (3) kindly provided by B. Hogan and by A. M. Colberg-Poley and P. Gruss, respectively. Hybridization was in 50% (vol/vol) formamide, and 10% (wt/vol) dextran sulfate (Pharmacia), at 42°C for 18 hr, essentially according to ref. 23. Blots were washed twice in 600 mM NaCl/60 mM sodium citrate at room temperature, twice in 300 mM NaCl/30 mM sodium citrate/0.1% $NaDodSO_4$ at 42°C, and twice in 45 mM NaCl/4.5 mM sodium citrate/0.1% $NaDodSO_4$ at 55°C. Autoradiographic exposure using Kodak X-Omat films was for the periods of time indicated in the figure legends.

Indirect Immunofluorescence. Cells were grown on 0.1% gelatin-coated glass (for PCC7 cells) or tissue culture plastic coverslips (for C1003 cells). Stage-specific mouse embryonic antigen (SSEA-1) (24) and tetanus toxin binding sites (25) were revealed on cells fixed with 3% paraformaldehyde (10 min at room temperature). Mouse monoclonal anti-SSEA-1 antibody was generously provided by D. Solter (24). Rabbit anti-mouse IgM conjugated with fluorescein isothiocyanate (FITC) was purchased from Nordic Immunology (Tilburg, The Netherlands). Tetanus toxin and rabbit anti-tetanus toxin antiserum were purchased from Wellcome. Goat anti-rabbit Ig-FITC was purchased from Nordic Immunology.

RESULTS

Neuronal Differentiation of C17S1 EC Cells, Clone 1003 (C1003 Cells). C1003 cells (Fig. 1*A*) respond to serum deprivation and growth for 4–5 days in defined medium (17) by differentiating essentially into neuroepithelial derivatives (Fig. 1*C*), which subsequently give rise to well-characterized cholinergic neurons (26, 27) with few epithelioid cells and rare fibroblast-like cells.

Treatment of C1003 cells with defined medium supplemented with retinoic acid (0.1 μM) had not been described before. It induces the cells to rapidly (2–3 days) differentiate into mainly neuron-like derivatives (Fig. 1*E*). Fig. 1*E* shows a C1003 culture grown for 2 days in these conditions. Neuron-like cells bearing long processes appear and form aggregates from which very long thick fibers extend later on. Extensive neural differentiation in the presence and in the absence of retinoic acid was shown by the fact that most of the cells had lost SSEA-1 (24) from their surface (Fig. 1 *A–F*), possessed tetanus toxin binding activity (25) (Fig. 1 *G–L*), and showed the accumulation of neurofilament-specific transcripts (28, 29) (Fig. 2). According to morphological criteria, differentiation progresses faster in the presence of retinoic acid than when serum-free medium is used alone. The tetanus toxin binding sites and the NF68 neurofilament transcripts also appear more rapidly when cells are grown in retinoic acid-supplemented defined medium than when they are grown without retinoic acid: C1003 cells have to be deprived of serum for 5 days to quantitatively express the same features of terminally differentiated nerve cells as cells grown

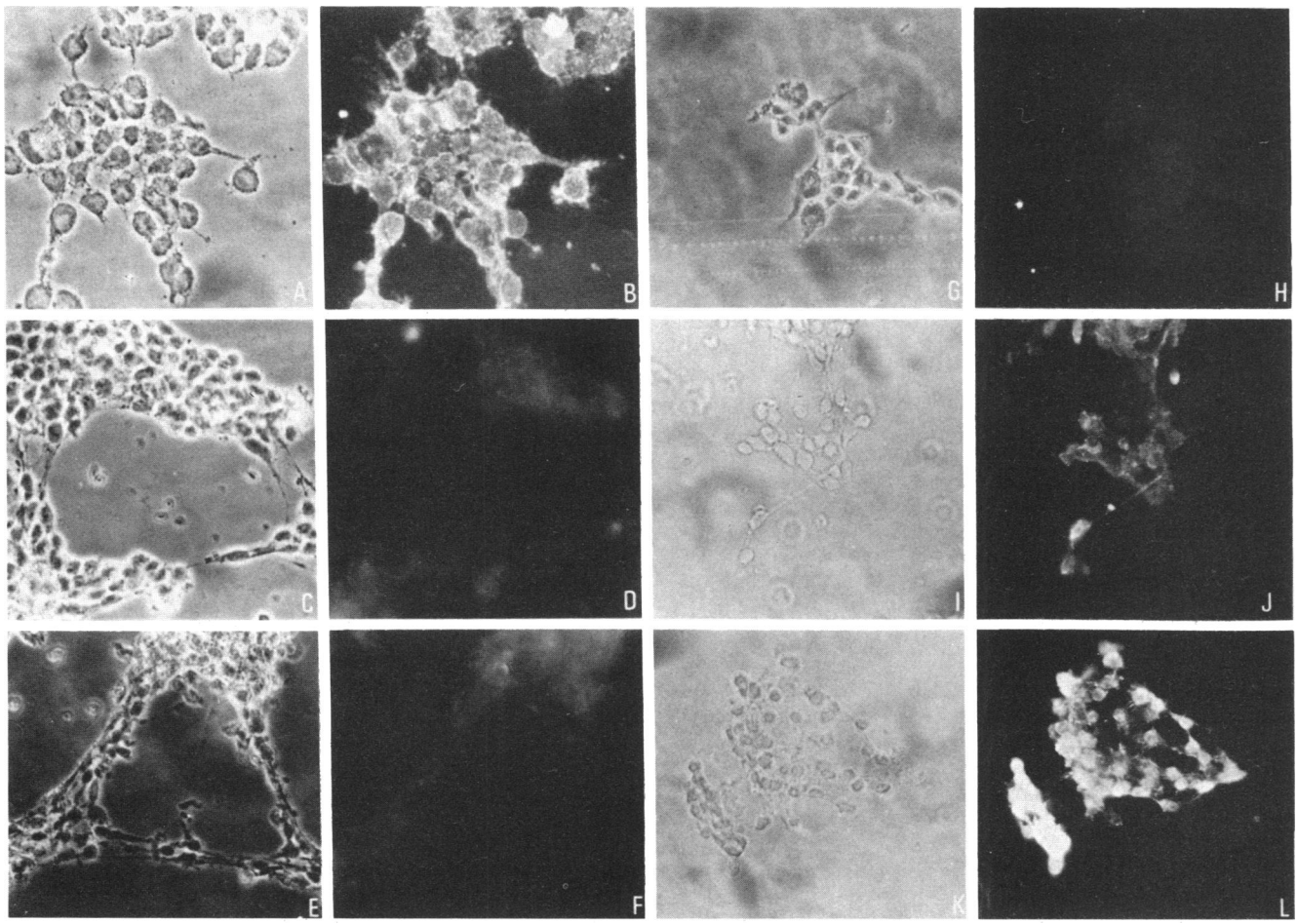

FIG. 1. Characterization of C1003 EC cells and their differentiated derivatives by indirect immunofluorescence. (*A–F*) Expression of SSEA-1 (21). (*G–L*) Expression of tetanus toxin binding sites (25). (*A, B, G, H*) C1003 EC cells. (*C, D, I, J*) C1003 cells grown for 4 days in serum-free medium. (*E, F, K, L*) C1003 cells grown for 2 days in serum-free medium supplemented with retinoic acid (0.1 μM). (*A, C, E, G, I, K*) Phase-contrast photographs. (*B, D, F, H, J, L*) Epi-illumination fluorescence. (All approximately ×200.)

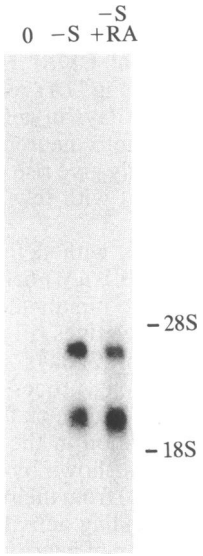

FIG. 2. Detection of neurofilament NF68 transcripts in poly(A)$^+$ RNA from C1003 EC cells (0), C1003 deprived of serum for 5 days (−S), and C1003 cells grown for 2 days in serum-free medium supplemented with retinoic acid (0.1 μM) (−S +RA). The probe used was a nick-translated 300-base-pair (bp) *Pst* I DNA fragment from the mouse NF68 cDNA clone (28), coding for the 68-kilodalton neurofilament protein. It detects two RNA species, 2.5 and 4.0 kb, transcribed from the same gene (29). Positions of rRNA markers are shown on the right.

for 2 days in serum-free medium in the presence of retinoic acid (Fig. 1 *J*, *L* and Fig. 2).

Poly(A)$^+$ RNA was isolated from cell samples harvested at various times during differentiation in the presence or in the absence of retinoic acid and RNA blots were hybridized with the homeobox-containing *H24.1* DNA probe (8). No hybridizing mRNA was observed in C1003 cells during the course of their differentiation in serum-free medium (Fig. 3*A*), whereas clear and strong hybridization was detected when retinoic acid had been added to the differentiation medium (Fig. 3*B*). In addition to the main 2.1-kilobase (kb) hybridizing RNA, two mRNA bands are visible; the largest of them follows slower induction kinetics. The relationship between these two RNAs and the 2.1-kb RNA is not understood yet.

Differentiation of C1003 Cells in the Presence of Serum. Retinoic acid induces the C1003 EC cells in serum-containing medium to form a population of mainly flat endoderm-like cells with a few neural derivatives growing on top of the flat cells (not shown). A blot containing equal amounts of poly(A)$^+$ RNA isolated from cells harvested at various time intervals after retinoic acid addition was hybridized with the *H24.1* homeobox probe. As in the case of the C1003 neural differentiation induced by serum withdrawal in the presence of retinoic acid, a strong accumulation of homeobox-specific transcripts was observed (Fig. 3*C*).

The response of the H24.1 gene to retinoic acid is very rapid, being detectable as soon as 3 hr after retinoic acid addition (Fig. 3*D*). Maximal amounts of the 2.1-kb hybridizing transcripts were detected by 1 day after induction. This level then remained constant for at least 7 days (data not shown).

Differentiation of PCC7-S.Aza R1 EC Cells, Clone 1009 (PCC7 Cells) in the Absence or in the Presence of Retinoic Acid. PCC7 EC cells (Fig. 4*A*) have been reported to differentiate into well-characterized cholinergic neuronal derivatives upon either one of two alternative treatments: aggregation and subsequent plating of the aggregates (30) or retinoic acid (0.1 μM) treatment of a EC cell monolayer (19). Neurite-like processes (Fig. 4 *B*, *C*), cholinergic neurotransmitters, and

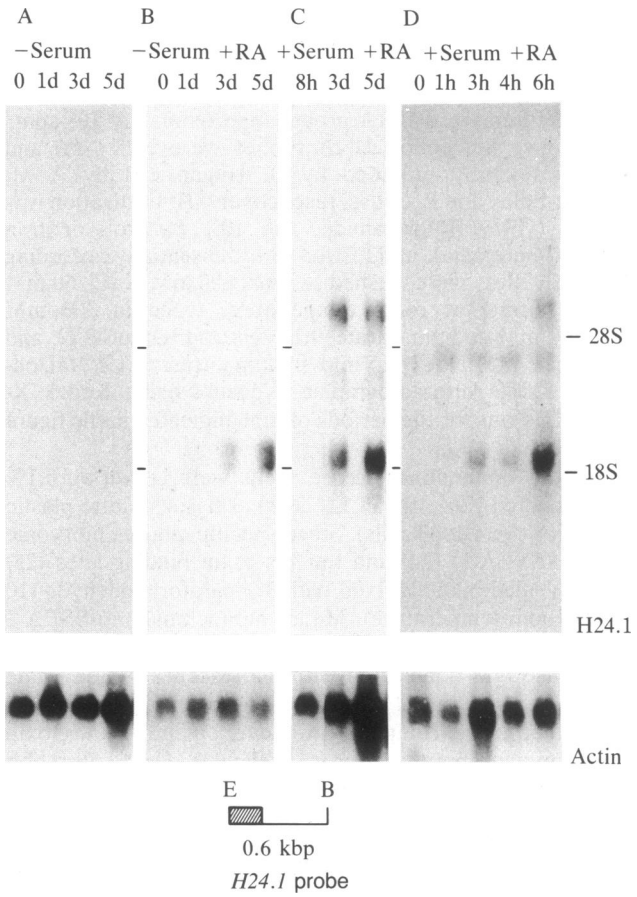

FIG. 3. Detection of H24.1 homeobox-related transcripts in C1003 EC cells and their differentiated derivatives. (*A*) Poly(A)$^+$ RNA from C1003 EC cells (0) and C1003 cells deprived of serum for 1, 3, and 5 days. (*B*) Poly(A)$^+$ RNA from C1003 EC cells (0) and their differentiated derivatives grown for 1, 3, and 5 days in serum-free medium supplemented with retinoic acid (RA) (0.1 μM). (*C*) Poly(A)$^+$ RNA from C1003 cells induced to differentiate by retinoic acid (1 μM) in serum-containing medium for 8 hr, 3 days, and 5 days. (*D*) Poly(A)$^+$ RNA from C1003 EC cells (0) and from C1003 cells induced to differentiate by retinoic acid (1 μM) in serum-containing medium for 1 hr, 3 hr, 4 hr, and 6 hr. *A*, *B*, and *C* are parts of a same experiment; *D* is a separate experiment. Exposure time was 5 days for *A* and *D* and 3 days for *B* and *C*. Hybridization of the filters with a β-actin mRNA probe is shown below each panel. The structure of the *H24.1* probe is depicted at the bottom. The hatched area represents the homeobox. E, *Eco*RI restriction site; B, *Bam*HI restriction site; kbp, kilobase pairs.

intermediate neurofilaments appear sooner in the case of retinoic acid-treated cells (3–4 days) than they do in the aggregated cultures (7–8 days).

Most of the cells induced to differentiate according to the two protocols were shown to have lost SSEA-1 (data not shown). Hybridization experiments using poly(A)$^+$ RNA isolated from cells treated with retinoic acid for 3 days revealed a 2.1-kb mRNA species hybridizing with the *H24.1* homeobox probe (Fig. 5*A*). In contrast, poly(A)$^+$ RNA isolated after 4-day-old cell aggregates had been plated for different periods of time did not give any hybridization signal with the H24.1 probe. Fig. 5*A* shows the result obtained from aggregates plated for 4 days. Hybridization with a β-actin probe proved that nearly equal amounts of intact RNA were present in each lane (not shown).

Differentiation of PSA-1-NG2 EC Cells (PSA-1 Cells) in the Presence or in the Absence of Retinoic Acid. The PSA-1-NG2 EC cell line (20) (Fig. 4*D*) is a highly pluripotent cell line that differentiates after the cells have been allowed to form aggregates and to grow in suspension for some time. These

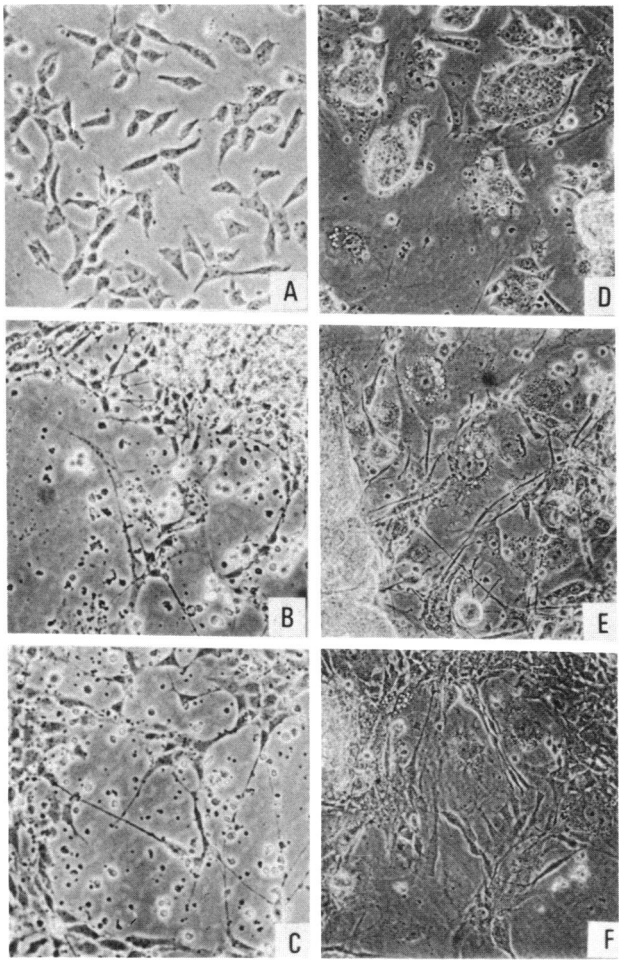

FIG. 4. Phase-contrast photographs of EC cells and their differentiated derivatives. (*A*) PCC7 EC cells. (*B*) PCC7 cells grown for 4 days as aggregates in serum-containing medium and subsequently allowed to attach to tissue culture dishes for 4 days. (*C*) PCC7 cells grown for 3 days as a monolayer in serum-containing medium supplemented with retinoic acid (0.1 μM). (*D*) PSA-1 cells grown without feeder layer for 2 days (aggregate formation stage). (*E*) PSA-1 cells grown as in *D* for 3 days; aggregates were then grown for 4 days in Petri dishes and subsequently plated on tissue culture surface for 3 days. (*F*) Same as *E*, except that retinoic acid (1 μM) was present as soon as the aggregates were transferred into Petri dishes. (All approximately ×125.)

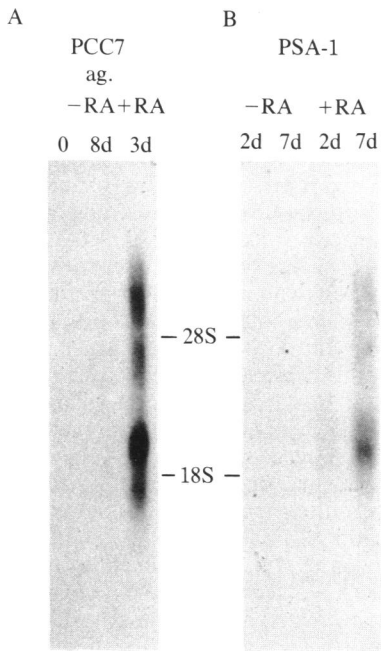

FIG. 5. Hybridization with nick-translated *H24.1* DNA. (*A*) Poly(A)$^+$ RNA from PCC7 EC cells, from PCC7 cells grown as aggregates for 4 days and subsequently plated onto tissue culture surface for 4 days, and from a monolayer of PCC7 cells treated with retinoic acid for 3 days. (*B*) Poly(A)$^+$ RNA from PSA-1 cells at two different stages of their spontaneous differentiation: 2d, aggregate formation, 2 days after plating the EC cells without feeder layer; 7d, differentiated cells arising 4 days after plating 3-day-old aggregates on tissue culture surface. Cells for the two lanes on the right were cultured in the presence of retinoic acid (1 μM).

aggregates develop similarly to mouse blastocysts: after they have been plated on a tissue culture surface, many differentiated derivatives appear, similar to those found in gastrulating embryos (20, 21, 31). Several investigators, using different isolates of the same *H24.1* mouse homeobox gene (5, 6, 8), have shown that this homeobox locus is expressed in developing mouse embryos (5, 6, 8), as soon as day 7.5 *post coitum* (8). However, no *H24.1* transcript was detected at any stage of PSA-1 cell differentiation (6). To investigate whether the addition of retinoic acid during PSA-1 aggregation and differentiation would give rise to accumulation of *H24.1* transcripts we treated PSA-1 EC cells with retinoic acid (1 μM) for different periods of time while they were induced to differentiate.

Clear differentiation was visible 3 days after plating the PSA-1 cell aggregates, both in the presence and in the absence of retinoic acid (Fig. 4 *E, F*), many similar types appearing in the two cell populations. Fig. 5*B* shows that *H24.1* transcripts were detected only in RNA that originates from retinoic acid-treated cultures. A strong accumulation of *H24.1* transcripts occurred when retinoic acid was present from the initial moment of induction of differentiation (plating of the cells without feeder layer) as well as when retinoic acid was added much later (not shown).

DISCUSSION

Activation of *Antp*-related homeobox-containing genes to high levels of expression has been reported to occur during differentiation of EC cells. These observations were made on EC cells induced to differentiate after treatment with a chemical agent, usually retinoic acid, that often triggers the generation of a restricted spectrum of tissue derivatives. Such a method is obviously very useful for studying commitment of pluripotent stem cells to acquire particular differentiated characteristics and for analyzing the sequential events leading to differentiation. However, the biological relevance of chemical induction of EC cells should be critically evaluated when this system serves as a model for stem cell differentiation during early mouse embryogenesis. High levels of expression of a particular homeobox gene (*Hul*, homolog of the mouse *H24.1* gene) have been observed during retinoic acid-induced differentiation of a human teratocarcinoma cell line (6) but not during spontaneous differentiation of highly pluripotent mouse EC (6) and EK (8) cells into derivatives of the three germ layers. Induced accumulation of large amounts of homeobox-containing gene transcripts might have resulted from a direct effect of retinoic acid. In favor of this possibility are the data reported by Hauser *et al.* (6), who showed a dependence of *Hul* homeobox gene expression on the continuous presence of retinoic acid during differentiation of NT2/D1 human teratocarcinoma cells. We have therefore asked whether the induction of homeobox genes to high levels of expression occurs during EC cell differentiation stimulated by both chemical and nonchemical methods. In the experiments described above, accumulation of large amounts of homeo-

box transcripts correlates with retinoic acid-induced EC cell differentiation rather than with cell differentiation as such: expression of the *H24.1* homeobox gene is not detected when serum deprivation induces the C1003 cells to differentiate along the neuronal pathway; the presence of retinoic acid during differentiation of these cells along a pathway shown to be similar to the former, according to morphological and biochemical criteria, leads to a strong accumulation of homeobox transcripts. Our results mean that differentiation until the stages we describe may occur without the concomitant accumulation of abundant *H24.1* transcripts detected in the presence of retinoic acid. Interestingly, addition of retinoic acid to C1003 cells already engaged in differentiation by being grown for 3 days in serum-free medium also leads to accumulation of *H24.1* transcripts (not shown). Differentiation of PSA-1 EC cells into various derivatives also occurs without concomitant accumulation of high levels of *H24.1* gene transcripts. This suggests that this accumulation is a direct effect of retinoic acid and does not correlate with EC cell differentiation.

The expression of *Antp*-type homeobox genes other than *H24.1* was also followed in all or some of our experiments (data not shown). Transcripts from the *m6-12* homeobox gene are not detected during neuronal differentiation of C1003 cells in serum-free medium. As described above for *H24.1* gene expression, the presence of retinoic acid in the defined medium stimulates the accumulation of m6-12 as well. A very high level of *m6-12* expression is also observed in serum-containing medium supplemented with retinoic acid. This level remains high for at least 7 days, provided retinoic acid is kept in the differentiation medium. A recently isolated *Antp*-related homeobox-containing gene (F.M. and J.D., unpublished data), different from *m6-12* and *H24.1*, gave similar results when used as a probe to detect transcripts in all three EC cell lines used in this work: a strong accumulation of transcripts is detected only when the cells have been induced to differentiate either by or in the presence of retinoic acid. High levels of expression of this homeobox gene and of *m6-12* thus also do not seem to be necessary for EC cell differentiation along the pathway we have looked at. The abundance of the transcripts might result from some direct effect of retinoic acid.

Induction to high levels of expression of homeobox genes by retinoic acid is not a general phenomenon. Mouse fibroblasts are known to respond to retinoic acid: they show an increased synthesis of extracellular matrix proteins (32). No expression of the *H24.1* homeobox gene was detected in retinoic acid-treated Swiss 3T3 fibroblasts (data not shown). In our experiments performed thus far, only differentiating EC cells seem to respond to retinoic acid by a strong accumulation of homeobox transcripts.

Our results do not rule out that the *Antp*-related homeobox genes we have considered might be expressed at an extremely low level when EC cells differentiate in the absence of chemical inducer. Very few copies of homeobox transcripts could remain undetected in our blot analysis as they might have remained undetected in the work of Hauser *et al.* (6). In favor of such a possibility are the experiments reported by Jackson *et al.* (8), who detected a very low level of *H24.1* gene expression long after they had induced EK cells to differentiate in the absence of any chemical inducer. Expression of homeobox-containing genes considered here, even at an extremely low level during stem cell differentiation in the absence of chemical inducer, would be consistent with a role of these genes during cell differentiation and pattern formation in early embryos.

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