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SPECIFIC EXPRESSION OF HOMOEOBOX-CONTAINING GENES DURING INDUCED DIFFERENTIATION OF EMBRYONAL CARCINOMA CELLS

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SUMMARY - A teratocarcinoma stem cell line, P19S1801A1, differentiates after induction with drugs (retinoic acid or dimethyl sulfoxide) in aggregate cultures as outgrowths in vitro. We have used this model of murine embryonic differentiation to show that several homoeobox-containing genes are newly activated while others remain active throughout. Transcripts of 1.5, 1.7, 1.8, 3.9, and 5.0 kb were detected using two different DNA probes. Some of these transcripts were stage-specific but not cell-type specific. Affinity-purified antibodies to a synthetic peptide also showed the appearance of nuclear antigen in the embryonal carcinoma stem cells and in differentiated cell types in heterogeneous patterns. This study illustrates the usefulness of teratocarcinoma model systems to analyze homoeobox gene activation during embryonic development and differentiation.

INTRODUCTION - The availability of embryonal carcinoma cell lines has greatly facilitated studies of the mechanisms of cell differentiation in vitro. Various cell lines are known to differentiate into several cell types in vitro after an appropriate chemical treatment. One of these (P19S1801A1, referred to as 01A1) is an ouabain and thioguanine resistant clone of the euploid pluripotent embryonal carcinoma cell line, P19, and has the potential to differentiate into muscle or neural cells after particular experimental manipulation (1,2).

Recently, members of a certain set of genes that are known to control embryonic development and differentiation in Drosophila were found to contain a highly conserved region, the homoeobox (3). The fact that the homoeobox (180 bp) sequence has also been found in the genome of several species, including mammals, has prompted the speculation that similar genes (homoeobox-containing genes) would play significant roles during development and differentiation in mammals (4,5). To test this hypothesis, embryonal carcinoma cell lines have been proved to be very valuable. Specific transcripts that seem to

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be present only after differentiation of F9 cells are induced by retinoic acid (6). In the present study we have used two homoeobox-containing sequences in order to study expression patterns during the differentiation of 01A1 to cardiac muscle induced by DMSO or to neural and glial cells induced by retinoic acid in vitro (1,2).

MATERIALS AND METHODS

Cells and treatments. P19S1801A1 (01A1) cells were used in this study. The cell line was obtained from Dr. M. McBurney, of the Department of Medicine, University of Ottawa, Canada. They were cultured in α-MEM containing 10% fetal calf serum and maintained as described (1,2). The cells were treated with 0.5% dimethyl sulfoxide (DMSO) or 10^{-6}M retinoic acid (RA) in different series of experiments. The cells were cultured as aggregates for 5 days in petri dishes, then they were placed in tissue culture dishes for 6 more days. Cultures were terminated at different time intervals, 3 days in petri dishes (3/0); 5 days in petri dishes (5/0); 3 days in tissue culture dishes (5/3); and 6 days in tissues culture dishes (5/6).

RNA isolation. After the termination of each experiment the cell pellet was dissolved in 2 ml of 4M guanidine thiocyanate, 25 mM sodium citrate, pH 7.0, 10 mM EDTA, and 0.5% laurylsarcosine, and 2-mercaptoethanol was added to 1%. The samples were homogenized with a motor-driven teflon pestle. The homogenate was layered on 2 ml of 1 g/ml CsCl in 0.03 M sodium acetate, pH 5.2. Samples were centrifuged at 40,000 rpm for 18 hr at 20°C. After discarding the supernatant the pellets were resuspended in 10 mM Tris, pH 7.5, 5 mM EDTA, 1% SDS, and extracted with an equal volume of 4:1 chloroform:butanol. RNA was ethanol precipitated after the addition of 0.1 volume of 3 M sodium acetate pH 5.2. The pellet was rinsed with 70% ethanol and dissolved in the appropriate volume of H_2O.

Northerns. The RNAs (40 μg) were run on a 1.2% agarose gel in MOPS (3-[N-morpholino] propane-sulfonic acid, Sigma Chemical Company) and 2.2 M formaldehyde. The RNA and gels were prepared for electrophoresis essentially as described by Maniatis et al (7). After electrophoresis, gels were denatured in 50 mM NaOH, 10 mM NaCl for 45 min, neutralized by soaking in 0.1 M Tris-Cl, pH 7.5, for 45 min, and finally soaked in 20 x SSC for 1 hr. The transfer to nitrocellulose filters was accomplished with 20 x SSC. The filters were then washed briefly in 6 x SSC, air dried at room temperature, and baked in a vacuum oven for 2 hr at 80°C.

Probes. Two mouse homoeobox-containing genes were used to probe the RNAs. pMo-10 and m6-12 were kindly provided by Dr. F.H. Ruddle, of Yale University and Drs. A.M. Colbey-Poley and P. Gruss, Heidelberg, Federal Republic of Germany, respectively. Both of these probes were isolated from mouse genomic libraries (5,6). pMO-10 is a 1.4 kb Eco RI fragment containing the homoeotic homology at the 3' end. m6-12 is a 1.9 kb Eco RI fragment from a mouse genomic library. Both have striking degrees of homology with Drosophila Antp, ftz, and Ubx genes. The probes were nick-translated with α-^{32}PdCTP (New England Nuclear) and hybridized to the filter as described (8).

Immunohistochemistry. A synthetic peptide from a highly conserved region of the homoeobox genes containing 13 amino acids was prepared for us by Dr. H. Weingarten of the Monsanto Company, St. Louis, Missouri. From the amino-terminal end: Arg-Gln-Ile-Lys-Ile-Trp-Phe-Gln-Asn-Arg-Arg-Met-Lys. This was linked to keyhole limpet hemocyanin (KLH) and used to immunize rabbits. All these procedures have been described earlier (9). Affinity purified anti-

bodies were prepared by adsorption to the peptide linked to AH-Sepharose (Pharmacia). Immunoperoxidase staining using avidin-biotin complex was used as described by the manufacturers (Vector Laboratories, CA). Cells were grown on coverslips and at appropriate times were fixed in 3.7% formaldehyde in phosphate buffered saline (PBS). After quenching in 0.1 M glycine-PBS, the coverslips were treated with methanol containing 1.3% H_2O_2 to quench endogenous peroxidase and to ensure that antibodies would penetrate the cells. Primary antibody incubations were for 16 hr at 4^0 using 30 µg/ml affinity-purified antibodies. Other procedures were as described earlier (9).

RESULTS AND DISCUSSION

When O1A1 cells are treated with 0.5% DMSO as aggregate cultures in petri dishes for 5 days and subsequently transferred to tissue culture dishes, they differentiate predominantly into cardiac muscle. Under the same culture conditions with 10^{-6} M RA, they differentiate into neurons and glial cells that become evident after 2 days in tissue culture dishes (1,2). Differentiation and the appearance of muscle cells in DMSO-treated cultures start at about the fifth to sixth day after plating in tissue culture dishes. No differentiation occurs without both drug treatment and aggregation (1,2).

The expression pattern of Mo-10 and m6-12 during the process of aggregation and differentiation is shown in Figure 1. When Mo-10 is used as a probe, accumulation of several transcripts occurs during the 11-day period. Transcript sizes are about 1.5 kb, 1.7 kb, 1.8 kb, 3.9 kb and 5.0 kb and become maximal in 5/3 samples. When m6-12 is used as a probe, the 1.7 kb and 5.0 kb transcripts predominate in all series and are highest by 5/3 days, after which expression decreases slightly. The 5.0 kb fragment co-migrates with the 28S rRNA. The same phonomenon was observed by Hart *et al* (10) using a different probe containing homoeobox sequences, and it could be attributed either to a homology between the sequences of these probes and 28S rRNA or to a homoeobox-containing transcript which co-migrates with 28S-rRNA. A similar sized transcript of 5.1 kb has been detected with this probe in testis RNA (11). The 1.7 kb transcript detected by the m6-12 probe is not 18S, because this rRNA species can be seen on stained gels to run more slowly than the 1.7 kb band. The M6-12 probe detects a 1.5 kb transcript in P19 EC cells (from which O1A1 cells were derived) that is likely to be the same as our 1.7 kb transcript run under slightly different electrophoretic conditions (6).

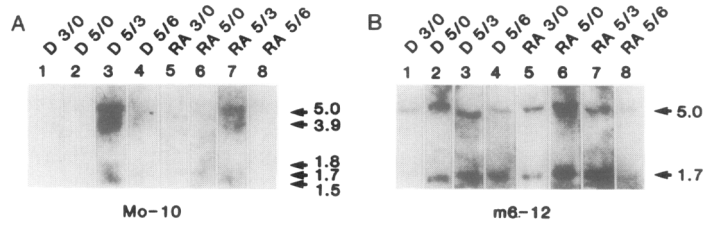

Figure 1. Northern blot hybridization of RNA from different experimental series (see Materials and Methods sections) analyzed with Mo-10 (A) and m6-12 (B) probes. The same filter was used in the two hybridizations. D: DMSO; RA: retinoic acid. Equal amounts of RNA were used for each sample (40 µg), and inspection of the ethidium bromide-stained gel confirmed this and indicated that no samples were degraded.

Transcripts detected by Northern transfer may be translated into protein(s). Figure 2 shows that 01A1 stem cells tested for the presence of homoeobox sequence proteins using the sensitive immunohistochemical technique

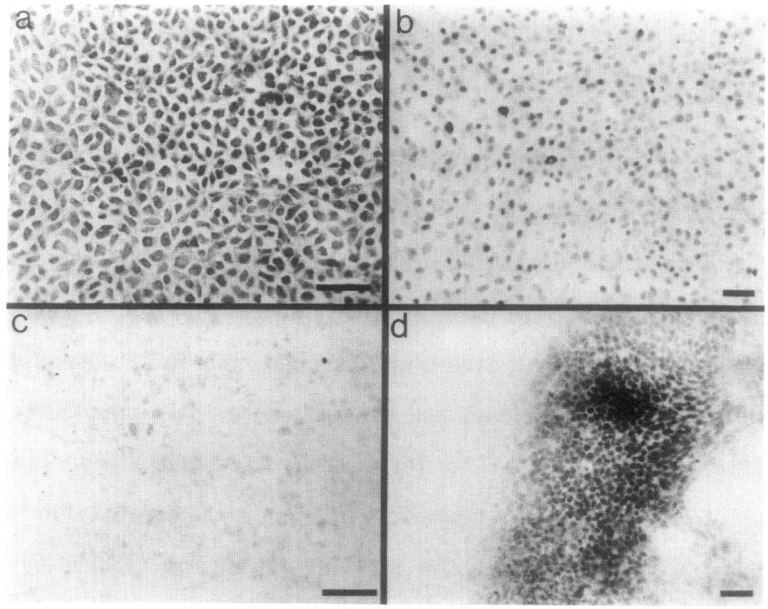

Figure 2. Immunohistochemical localization of proteins containing homoeobox sequences. 01A1 cell outgrowths stained by the avidin-biotin peroxidase technique using a rabbit antibody raised to a synthetic peptide. a) 01A1 stem cells on day 5/3 of aggregate outgrowths without drugs. All the nuclei are stained. b) Outgrowths on day 5/5 after retinoic acid induction. Fibroblast-like cells are shown with nuclear staining in about 50% of the cells. Stained mitotic chromosomes are also visible. c) Day 5/3 of outgrowth after induction with 0.5% DMSO. Little or no staining is present at this time. d) Day 5/5 of outgrowths induced with 0.5% DMSO. A clump of small cells with positively-stained nuclei is present in a field of larger negative cells. Bar = 50 microns.

of ABC peroxidase staining (see the Materials and Methods section) show specific nuclear reaction (Fig. 2a). The reaction is proportional to antibody concentration and is inhibited by the presence of purified peptide antigen. A nuclear localization has also been demonstrated for protein product in Drosophila (12). In 01A1 cells that differentiate following induction by retinoic acid (Fig. 2b) or 0.5% DMSO (Fig. 2c and d), a heterogeneous array of nuclear stained cells was observed. Some of the more interesting patterns are shown. Not all the cells in Figure 2b are stained, suggesting a cell-cycle dependence of reaction with the antibody. Cells stimulated with DMSO in Figure 2c are largely negative, while two days later intensely positive colonies in a negative field can be seen (Fig. 2d). We have not yet identified specific cell types that contain homoeobox proteins, since several simultaneous transcripts are observed to accumulate in these mixed differentiated cell types. Attempts to use the antibody to immunoprecipitate the native protein(s) were unsuccessful. Other techniques are currently being used to identify specific gene products.

In preparation for the identification of cell types that express homoeobox genes, we surveyed a number of embryonic, fetal, and adult tissues. The results illustrate that homoeobox proteins are ubiquitous, thus supporting reports of RNA transcripts in many murine tissues (13-15). Figure 3 shows some of the adult tissues that give a nuclear reaction in ABC-peroxidase tests with the antibody. Brain, kidney, and adrenal cortex give particularly strong reactions (Fig. 3a,d,f). Liver is less strong (Fig. 3g), and, like kidney, not all nuclei are reactive (compare Fig. 3g with hematoxylin and eosin stained, Fig. 3i). Reaction with the antibody is largely blocked by inclusion of the peptide in the reaction mixture, thus illustrating the specificity of the antibody (Fig. 3b,e,h). Other adult tissues that give nuclear staining with the antibody include testes but exclude cardiac muscle and spleen (Fig. 3c). Among embryonic and fetal tissues, 8th-day embryonic ectoderm and extraembryonic endoderm, fetal visceral yolk sac, and 12th day spinal cord were positively stained with the antibody (not shown).

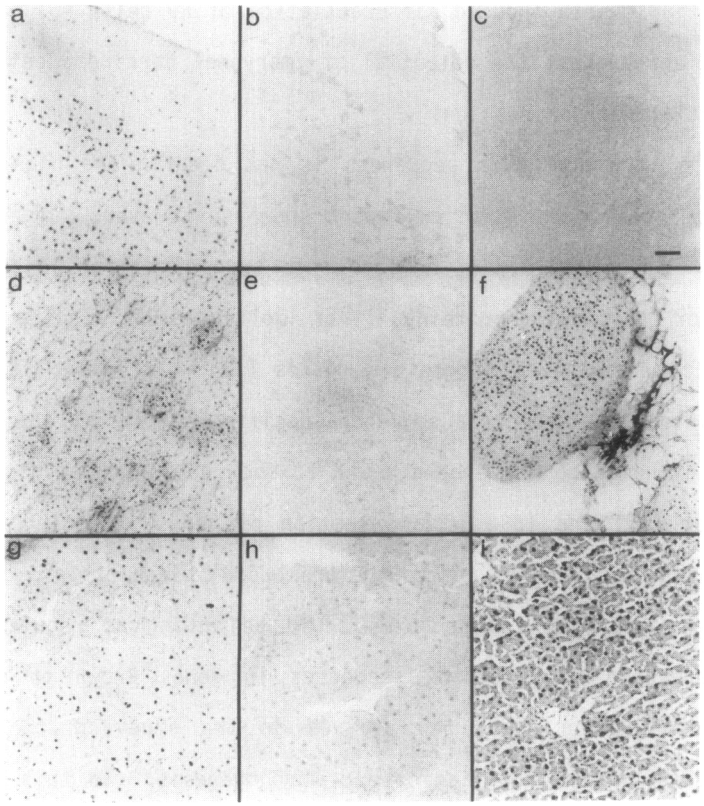

Figure 3. Immunohistochemical staining to show homoeobox proteins. See text for details of ABC-peroxidase staining: a) and b) adult mouse brain stained for homoeobox proteins in the absence (a) and presence (b) of blocking peptide. c) Adult mouse spleen is negative. d) and e) Kidney sections to show reaction (d) and reaction blocked with peptide (e). f) Adrenal cortex stained with anti-homoeobox. g) - i) Adult mouse liver, stained with anti-homoeobox antibodies (g); (h) is the same as (g) but in the presence of blocking peptide; hematoxylin and eosin staining (i). Bar = 50 microns.

In summary, when the Mo-10 probe is used, three transcripts of 1.5 kb, 1.6 kb, and 3.9 kb appear strongly but transiently after 5/3 days of treatment with both drugs, and their expression declines after this period (Fig. 1a), consistent with a differentiation stage-specific pattern of expression. The 1.7 kb and 5.0 kb transcripts detected with the m6-12 probe (Fig. 1b) do not seem to be differentiation-specific, but they could have general or housekeeping functions. We have found transcripts of similar sizes in mouse embryonic tissues (9,10, Tsonis and Adamson, unpublished data). The present data, however, strongly indicate that several homoeobox-containing transcripts may play roles in the differentiation process. Similar observations have been

made during retinoic-acid induced differentiation of F9 cells (6) and both of these observations suggest the potential of embryonal carcinoma cell lines to study differential gene expression.

The role of the homoeobox sequences is not clear. The high degree of conservation of this sequence in different species, however, suggests a very specific role. Some homology of homoeobox to type α2 mating locus of yeast (which codes for DNA binding proteins) first implicated DNA binding activities for the products of homoeobox-containing genes (16). Recently, it was shown that the engrailed gene encodes a sequence-specific DNA binding activity (12). Other regions of homoeobox have been found to show some homology to glucocorticoid receptor (17) and to a wide selection of genes including the switch region of immunoglobulin genes and to the primordial block of IgV_H genes (18). Such homologies imply that homoeoboxes could be acting as regulatory molecules. The presence of homoeobox sequences in genes known to control or regulate differentiation opens new avenues in the study of the molecular mechanisms underlying differentiation and morphogenesis. In this respect we feel that in vitro systems such as the one reported here will facilitate these studies.

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