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Differential expression of the homeobox gene *Hox-1.3* in F9 embryonal carcinoma cells

(homeotic genes/teratocarcinoma cells/transcription)

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ABSTRACT **The *Hox-1.3* gene is located on mouse chromosome 6 and has been previously shown to be expressed in mouse embryos and adults. In this study, we have examined the steady-state levels of the *Hox-1.3* transcripts in undifferentiated and differentiated F9 embryonal carcinoma cells. We find that there is a rapid increase of *Hox-1.3* transcripts after differentiation induction of F9 cells. The level of the major 1.85-kilobase (kb) transcript peaks at 16–24 hr after differentiation induction of F9 cells. By using primer extension techniques the 5′ ends of the major 1.85-kb transcript have been mapped to two sites in induced F9 cells. Cellular fractionation of RNA and transfer blot gel analysis has localized one minor transcript to the nucleus, whereas the major transcript and two additional minor transcripts appear in the nucleus and the cytoplasm of induced F9 cells. The results of nuclear run-off experiments with uninduced and induced F9 cell nuclei indicate that there is a substantial increase in the rate of *Hox-1.3* transcription upon induction of F9 cells with retinoic acid.**

The discovery of conserved DNA sequences, homeoboxes, in the coding region of genes that play a significant role in the developmental regulation of *Drosophila* (reviewed in refs. 1 and 2) has led investigators to examine other species for the presence of these sequences. In mouse, several clusters of homeoboxes have been found on different chromosomes (3–17). Transcripts containing some of these homeobox sequences appear in specific regions at precise times during murine development (7–11, 15, 16, 18–24). Other evidence supports the possibility that these genes may be of developmental importance in the mouse. A homeo domain of the Hox-1.5 mouse protein has been shown to bind to sequences 5′ to the *Hox-1.5* gene (25); antisera to a peptide within the Hox-1.3 protein (15) and to a peptide in the Hox-1.1 and Hox-1.3 proteins (26) demonstrate nuclear localization of these proteins by immunofluorescence, suggesting that, like their *Drosophila* counterparts, homeo domain proteins may function as nuclear regulatory proteins.

Because teratocarcinoma cells have been used to study related developmental changes in the early embryo, expression of these homeobox genes has been examined in undifferentiated embryonal carcinoma (EC) cells and their differentiated derivatives. By using the recently described nomenclature for the homeobox genes (27), the *Hox-1.1* (6, 11, 26, 28), the *Hox-1.2* (11), the *Hox-1.4* (23), the *Hox-3.1* (28), the *En-1* (4), and the *En-2* (22) genes have been reported to be expressed in teratocarcinoma cells. In some of these cases, several different-sized transcripts have been identified by probing with these genes. Because of sequence homologies between different homeobox genes (e.g., homologies outside of the homeobox region for the *Hox-1.3* and *Hox-2.1* genes; refs. 15, 21) careful examination of transcriptional patterns has awaited the cloning, sequencing, and sequence comparison of the homeobox genes. With the isolation, identification, and sequencing of the cDNA for the mouse *Hox-1.3* gene (15) we have been able to analyze the expression of this gene in undifferentiated and differentiated F9 cells by using probes unique to the mRNA.

In this report, we describe the rapid appearance of steady-state mRNA for the *Hox-1.3* gene upon differentiation induction of F9 cells with retinoic acid. Multiple *Hox-1.3* transcripts have been identified, the 5′ start sites of the major *Hox-1.3* mRNA have been mapped, and the rate of transcription of the *Hox-1.3* gene is compared in undifferentiated and retinoic acid-induced cultures of F9 cells.

MATERIALS AND METHODS

Cell Culture and Differentiation Conditions. F9 embryonal EC cells and NIH 3T3 cells were cultured as described (29). For differentiation studies, F9 EC cells were cultured in medium containing 0.5 μM retinoic acid, 0.1 mM isobutylmethylxanthine, and 1 mM dibutyryl adenosine 3′,5′-cyclic monophosphate (RAIC). Extreme care was taken to minimize the percentage of differentiated cells in our undifferentiated stock F9 cultures by filtering the cultures with 15-μm Nitex (Tetko, Elmsford, NY) cloth at each passage (30).

RNA Isolation. Total poly(A)$^+$ RNA was isolated according to the method of Vennstrom and Bishop (31). Cytoplasmic and nuclear RNAs were separated according to the methods of Favaloro *et al.* (32), and then poly(A)$^+$ was selected according to Vennstrom and Bishop (31). Mouse lung RNA was isolated according to the procedure of Chirgwin *et al.* (33).

RNA Transfer Blot Gel Analysis. Poly(A)$^+$ RNA was electrophoresed through a 1% agarose gel in 2.2 M formaldehyde as described (34). The gels were blotted onto nitrocellulose (Schleicher & Schuell BA 85). Hybridization of the filters with either nick-translated or random-primer labeled probes was done as described by Meinkoth and Wold (35). The blots were then exposed on Kodak XAR-5 film at −70°C in combination with intensifying screens.

Primer-Extension Analysis. Primer-extension analysis of poly(A)$^+$ RNA was performed as described by Colman (36) with minor modifications. An oligonucleotide, 5′-AATTGTTCGCTCACGGAACTATGATC-3′, complementary to bases 76–101 of Hox-1.3 (15) was 5′ end-labeled with [γ-^{32}P]ATP according to Davis *et al.* (37). The primer-extended products were electrophoresed (38) in parallel with the dideoxy sequence reaction products of a pBSM13 (Strat-

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Abbreviations: EC, embryonal carcinoma; RAIC, 0.5 μM retinoic acid/0.1 mM isobutylmethylxanthine/1 mM dibutyryl adenosine 3′,5′-cyclic monophosphate.

agene)-based plasmid template containing a 1.4-kilobase (kb) *Bgl* II fragment of the *Hox-1.3* gene, which begins 0.88 kb from the initiating ATG for the *Hox-1.3* gene. The plasmid template was primed with the unlabeled oligonucleotide used above, and dideoxy sequencing reactions were performed by using the Sequenase reagents (United States Biochemicals, Cleveland, OH).

Nuclear Isolation and Run-off Transcription. Nuclei from F9 EC and F9 RAIC-treated cells were isolated by a modification of the procedure of Levine *et al.* (39). For transcription reactions, 1.8×10^7 nuclei were incubated for 10 min at 26°C in a 200-μl reaction mixture containing 16% glycerol, 2.5 mM $MgCl_2$, 70 mM KCl, 2.5 mM dithiothreitol, 20 mM Tris·HCl (pH 8.3), 0.8 mM ATP, 0.4 mM CTP, 0.4 mM GTP, 8 μM UTP, and 250 μCi (1 Ci = 37 GBq) of [α-^{32}P]UTP (NEN; 600 Ci/mmol). ^{32}P-labeled RNA was purified extensively as described by Groudine *et al.* (40).

Hybridization of ^{32}P-Labeled RNA to Immobilized DNA. Plasmid DNAs were adsorbed to nitrocellulose filters (BA85) by using a slot-blotting manifold (39). Filters were then prehybridized overnight at 42°C in 50% formamide/5× Denhardt's solution (1× Denhardt's solution = 0.02% bovine serum albumin/0.02% Ficoll/0.02% polyvinylpyrrolidone), 0.75 M NaCl/75 mM sodium phosphate/5 mM EDTA/ 0.1% $NaDodSO_4$, and 100 μg of denatured salmon sperm DNA per ml. Equal amounts of radioactivity were added to the filters (the range for different experiments was 2.0–9.0 × 10^6 cpm/ml) and hybridized for 4 days at 42°C. ^{3}H-labeled M13 replicate form DNA was hybridized against unlabeled, immobilized M13 DNA as an internal control for relative hybridization efficiencies. The blots were washed four times for 15 min with 0.3 M NaCl/30 mM sodium phosphate/2 mM EDTA/0.1% $NaDodSO_4$ at room temperature and two times with 30 mM NaCl/3 mM sodium phosphate/0.2 mM EDTA/ 0.1% $NaDodSO_4$ at 50°C for 30 min. The filters were then subjected to autoradiography at −70°C with Lightning Plus image-intensification screens.

RESULTS

Induction of *Hox-1.3* Transcripts Upon Differentiation. The structure of the *Hox-1.3* gene is illustrated in Fig. 1 based upon previous work (15). In that study, an intron was identified 20 bp 5′ to the homeobox sequences in exon 2. The major transcript is 1.85 kb. Unless noted, RNAs examined were total poly(A)$^+$ RNA probed with the labeled fragment shown in Fig. 1. In our initial studies, RNA was examined from undifferentiated cells and cells in which differentiation was induced by RAIC. Since previous studies have shown that the level of *Hox-1.3* steady-state RNA is much greater in growing than in contact-inhibited fibroblasts (15), our experiments were performed to allow for continued growth of the teratocarcinoma cells to minimize the possibility of diminution of *Hox-1.3* mRNA because of growth inhibition. Fig. 2*A* illustrates a typical transfer blot gel analysis of RNA from undifferentiated and differentiated F9 cells. There is a rapid accumulation of a major 1.85-kb transcript within 24 hr of administration of RAIC. In addition, there are multiple larger transcripts from the RAIC-induced cells. A 4.0-kb mRNA that is synthesized in fibroblasts and a number of adult tissues (15) is also present in RAIC-induced F9 cell RNA. We also detect 3.1- and 5.0-kb minor transcripts in RAIC-induced F9 total poly(A)$^+$ RNA.

Because of the large increase of *Hox-1.3* RNA within 24 hr, similar studies were done for shorter times of induction (shown in Fig. 2*B*). The level of the 1.85-kb transcript dramatically increases within 4 hr of induction. In Fig. 3 we report the change in the amount of the *Hox-1.3* transcript (normalized against γ-actin RNA) as a function of time after induction. Densitometry of the γ-actin and *Hox-1.3* 1.85-kb transcripts was done taking care to choose exposures that were still within the linear range of autoradiographic exposure (this representation is based upon the assumption that the γ-actin mRNA levels are equivalent in the undifferentiated and differentiated cultures). The *Hox-1.3* RNA levels peak between 16 and 24 hr of RAIC administration and then decrease to ≈50% of the maximal level by day 2. Whether these changes are representative of all of the cells in the population or a combination of different subpopulations in the differentiating cultures remains to be determined. These changes in steady-state RNA levels are similar to those reported for *Hox-1.1* at early times of F9 differentiation (28) but differ at later times when there is a much sharper decrease of *Hox-1.1* mRNA.

Examination of the 5′ Ends of the Major *Hox-1.3* Transcripts. Primer extension analysis was performed on poly(A)$^+$ RNA from F9 EC cells, 24-hr RAIC-induced F9 cells, and adult mouse lung. The data are shown in Fig. 4. The sequencing ladder to the left is derived from dideoxy sequencing of the genomic sequences corresponding to the RNA so that specific start sites could be easily identified (see *Materials and Methods* for details). There are two major start site regions in RAIC-induced F9 cell RNA. The major proximal start site region covers bases −44 to −47 from what we believe to be the translational initiating codon (see ref. 15 for details of sequence). The major distal start site is at −74 from the initiating AUG of *Hox-1.3*. In adult mouse lung RNA there is one major start site region identical to the major distal start site from F9 EC cell RNA. There is a minor, proximal start site region identical to that for F9 EC cell RNA. Minor start sites present in both RNA preparations correspond to −78, −84/−85, −90/−91, −94/−95, and −106 to −108. In addition, RAIC-induced F9 cells had minor start sites at −136/−137. No clear signal was obtained from undifferentiated F9 cell RNA, as expected from the RNA transfer blot data of Fig. 2. It should be noted that the above positions assume that the 5′ noncoding *Hox-1.3* mRNA sequences are colinear with the genomic sequence previously described (15). Since the previously described cDNA (15) did not extend to these 5′ noncoding sequences, the possibility exists that the extreme 5′ ends of the transcripts are derived from sequences further upstream by splicing. However, no obvi-

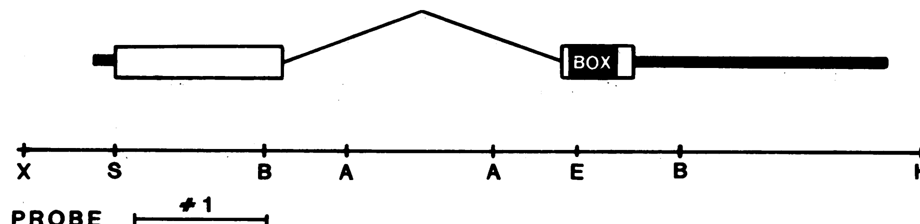

FIG. 1. Structure of *Hox-1.3* gene and major transcript (based upon ref. 15). The gene contains two exons separated by an intron 20 base pairs (bp) 5′ to the homeobox sequences. Solid thin line, noncoding region of the mRNA; BOX, the homeobox sequences; open boxes, the coding region of the mRNA. The restriction site locations on the genomic sequences are given below: X, *Xho* I; S, *Sac* I; B, *Bgl* II; A, *Aha* III; E, *Eco*RI; H, *Hin*dIII. The probe shown is a 450-bp fragment of the cDNA that was used in the RNA transfer blot gel analysis.

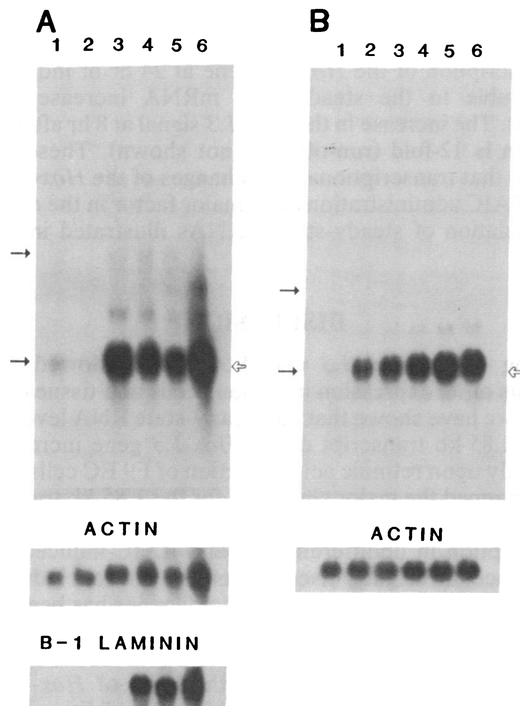

FIG. 2. Transfer blot analysis of total poly(A)$^+$ RNAs from F9 EC cells and NIH 3T3 fibroblasts. (*A*) Lane 1, NIH 3T3 fibroblast RNA; lane 2, F9 EC cell RNA; lanes 3–6, F9 EC cell RNA after 1 day (lane 3), 2 days (lane 4), 3 days (lane 5), and 4 days (lane 6) of RAIC induction. (*B*) F9 EC cell RNA after 0 hr (lane 1), 4 hr (lane 2), 8 hr (lane 3), 12 hr (lane 4), 16 hr (lane 5), and 24 hr (lane 6) of RAIC induction. Blots were rehybridized with a γ-actin probe (41) to compare RNA integrity and with a B1 laminin probe (42) to illustrate differentiation of the F9 EC cells under these conditions. The probe shown in Fig. 1 was used to analyze the blots. Gel conditions, RNA concentrations, and exposure times were as follows: (*A*) 1.2% agarose, 6.5 μg of RNA per sample, 7-day exposure; *Hox-1.3* probe, 4.8×10^8 cpm/μg; actin probe, 6.6×10^8 cpm/μg; B1 laminin probe, 6.0×10^8 cpm/μg. (*B*) 1.0% agarose, 8.5 μg of RNA per well, 60-hr exposure; *Hox-1.3* probe, 3.4×10^9 cpm/μg; actin probe, 1.5×10^9 cpm/μg. Closed arrows represent the positions of rRNA markers; open arrow represents the major *Hox-1.3*, 1.85-kb transcript.

ous splice acceptor consensus sequences could be identified for 900 bp 5′ to the putative start codon. When oligonucleotides complementary to genomic sequences 5′ and 3′ to the distal start site were used to probe F9 RAIC RNA by means of transfer blot analysis, the size and signal strengths of the

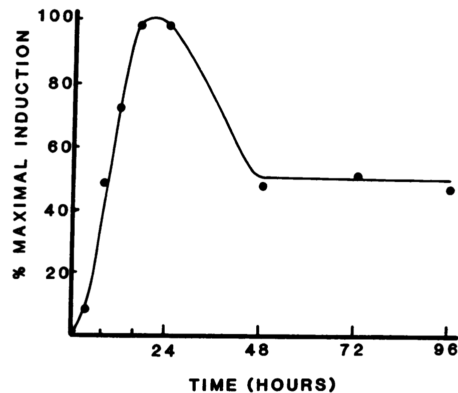

FIG. 3. Induction of 1.85-kb *Hox-1.3* RNA upon differentiation of F9 EC cells. Autoradiographic exposures of RNA transfer blots within the linear range of exposure were scanned with a densitometer and a ratio of *Hox-1.3*, 1.85-kb transcript/γ-actin transcript was calculated.

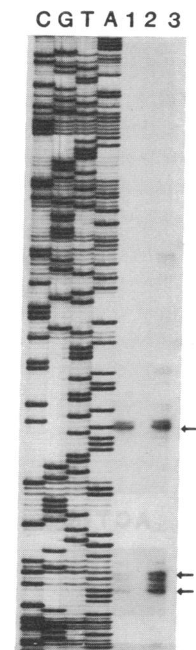

FIG. 4. Primer-extension analysis of *Hox-1.3* poly(A)$^+$ RNA from F9 cells, F9 RAIC-induced cells (24 hr of induction), and adult mouse lung. Lane 1, primer-extension products from 15 μg of adult mouse lung RNA; lane 2, 15 μg of undifferentiated F9 cell RNA; lane 3, 15 μg of RAIC-induced F9 cell RNA; lanes C, G, T, and A, sequencing lanes from a plasmid containing a 1.4-kb *Bgl* II fragment of the *Hox-1.3* gene including the region examined for primer-extension products here. Arrows correspond to the major 5′ ends of the extended products at −44 to −47 and −74 bases 5′ to the initiating codon of the *Hox-1.3* transcript.

transcripts identified were consistent with the above interpretation of the primer-extension experiments (data not shown). Furthermore, *Hox-1.3* transcripts from mouse lung are colinear with the genomic sequences between the AUG and cap site at −74 (J.G., unpublished observation).

It should be noted that in a previous report (11) the *Hox-1.2* transcript in 24-hr induced F9 cells was identified as having a size comparable with the *Hox-1.3* transcript we describe here. As noted by Kessel *et al.* (26), the probe used by Colberg-Poley *et al.* (11) to identify *Hox-1.2* transcripts in fact spans the *Hox-1.2* and *Hox-1.3* transcriptional units and likely detects both genes' products.

Localization of Multiple Transcripts Coded by *Hox-1.3*. A number of larger transcripts are detected when total poly(A)$^+$ RNA from RAIC-induced F9 cells is probed with a fragment specific to the *Hox-1.3* gene (see Fig. 2). These minor transcripts were examined to determine their cellular localization. Nuclear and cytoplasmic RNAs were isolated from F9 cells treated with RAIC for 24 hr and compared with total RNA. Transfer blot gel analysis of these poly(A)$^+$ RNAs reveals that the 3.1-kb transcript is present in the nuclear but not the cytoplasmic fraction (see Fig. 5, lanes 1 and 2). The size of this transcript and its cellular localization suggest that it may be the unspliced precursor to the major 1.85-kb transcript. The blots used in this experiment were stripped and rehybridized to a genomic fragment coding for the *Hox-1.3* intron. The intron probe hybridizes to the 3.1-kb transcript (data not shown). The 4.0-kb and 5.0-kb transcripts are present in nuclear and cytoplasmic fractions (Fig. 5, lanes 2 and 3) but are not detected with the intron probe (data not shown). This suggests that they may be functional transcripts.

Analysis of the Rate of *Hox-1.3* Transcription During F9 EC Cell RAIC Induction. The rapid accumulation of steady-state

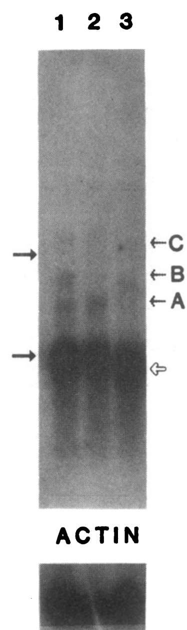

FIG. 5. Cellular localization of *Hox-1.3* transcripts. Total cytoplasmic and nuclear poly(A)$^+$ RNAs were isolated from F9 cells treated with RAIC for 24 hr. Seven and one-half micrograms of RNA per sample was then run on a 1% agarose gel, blotted, and probed with the *Hox-1.3* probe described in the legend to Fig. 1. Lane 1, total poly(A)$^+$ RNA; lane 2, nuclear poly(A)$^+$ RNA; lane 3, cytoplasmic poly(A)$^+$ RNA. The specific activity of the *Hox-1.3* probe was 1.2 $\times$ 10^9 cpm/μg, and the exposure time was 9 days. The specific activity of the γ-actin probe was 1.1 $\times$ 10^9 cpm/μg, and the exposure time was 48 hr. Solid arrows indicate positions of the 18S and 28S rRNA markers. The open arrow identifies the major 1.85-kb transcript. Letters refer to the positions of the minor transcripts: A, 3.1-kb transcript; B, 4.0-kb transcript; C, 5.0-kb transcript.

Hox-1.3 mRNA during F9 EC cell RAIC induction illustrated in Figs. 2 and 3 could be due to several factors. Nuclear run-off experiments were performed on nuclei from undifferentiated and 24-hr RAIC-induced F9 cells to investigate whether one of the controls is transcriptional. Representative results of these experiments are illustrated in Fig. 6. The signal obtained for *Hox-1.3* in undifferentiated nuclei (lane 1) is comparable to that of the plasmid control pSP65. However, upon RAIC administration for 24 hr, there is a dramatic increase in the signal obtained for *Hox-1.3* (lane 2). The rates of transcription of c-*myc* are similar for undifferentiated and RAIC-induced nuclei, as reported by others (45–47). The transcription rates of c-Ha-*ras* and γ-actin do not change appreciably during the two time points shown. Densitometric

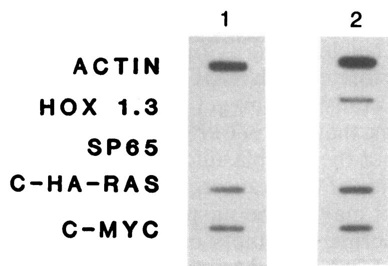

FIG. 6. Measurement of the rate of *Hox-1.3* transcription in F9 EC cells and cells induced with RAIC. Lane 1, F9 undifferentiated nuclei; lane 2, nuclei from 24-hr RAIC-induced F9 cells. The probes utilized were as follows: γ-actin (pHF1; ref. 41); Hox 1.3 [r-*Hox-1.3* Bam (r = recombinant), complete *Hox-1.3* cDNA inserted into the *Bam*HI site of pSP69]; c-Ha-*ras* (pbcNI; ref. 43); and c-*myc* (pSV-c-*myc*; ref. 44). Filters were hybridized with 7 $\times$ 10^6 cpm of ^{32}P-labeled RNA per ml. Autoradiographic exposure time was 45 hr.

analysis reveals that when the *Hox-1.3* transcriptional rates are normalized for c-*myc*, γ-actin, or c-Ha-*ras*, the increase in transcription of the *Hox-1.3* gene at 24 hr of induction is comparable to the steady-state mRNA increase (30- to 40-fold). The increase in the *Hox-1.3* signal at 8 hr after RAIC addition is 12-fold (run-off data not shown). These results suggest that transcriptional rate changes of the *Hox-1.3* gene upon RAIC administration are a major factor in the observed accumulation of steady-state mRNAs illustrated in Figs. 2 and 3.

DISCUSSION

Cloning of the *Hox-1.3* cDNA (15) has allowed for the analysis of its expression in various cells and tissues. In this report we have shown that the steady-state RNA levels of the major 1.85-kb transcript of the *Hox-1.3* gene increase dramatically upon retinoic acid induction of F9 EC cells, and we have mapped the major start sites for the 1.85-kb transcripts, identified cytoplasmic transcripts, and compared *Hox-1.3* transcription in undifferentiated and RAIC-induced F9 nuclei. Since the original discovery of retinoic acid induction of F9 cells (48) the expression of several genes has been shown to be affected by this reagent. Visible morphological changes in F9 cells require 2–3 days of retinoic acid induction (48). In contrast, dramatic increases in the level of *Hox-1.3* transcripts are observed within 4 hr after the addition of RAIC (Fig. 2*B*). In other experiments we have detected increased levels of transcripts as early as 2 hr after induction (data not shown). These observations, along with similar observations by Breier *et al.* (28) for *Hox-1.1* gene expression, suggest that the *Hox-1.1* and *Hox-1.3* gene products may have a very early function in the differentiation process in F9 cells. However, a recent report indicates that an alternative pathway leading to these changes occurs without the involvement of homeobox transcripts (49) in two different EC cell lines.

Fig. 3 illustrates the changes in the *Hox-1.3* major transcript in differentiating cultures of the F9 cells. There is a very sharp increase in transcript peaking between 16 and 24 hr of induction. In the next 24 hr the transcript levels fall off and remain at ≈50% of the maximal level. At the present time we do not know if this falloff represents the pattern for all of the cells in the culture or an average of subpopulations of cells in these differentiating cultures. Though this is similar to the pattern found by Breier *et al.* (28) for *Hox-1.1* regulation in F9 cells, in that study the level of *Hox-1.1* RNA fell more sharply than that detected here for *Hox-1.3*.

We have identified the minor 3.1-kb transcript as a nuclear transcript (Fig. 5). This is most probably the unspliced precursor to the 1.85-kb transcript. We have also detected in cytoplasmic fractions the 4.0-kb and 5.0-kb transcripts. Although these transcripts clearly contain *Hox-1.3* sequences (given the probe used), their complete sequence is yet to be determined.

Nuclear run-off analysis (Fig. 6) reveals that a primary factor in the accumulation of the major *Hox-1.3* transcript upon RAIC induction of F9 cells (Figs. 2 and 3) is a dramatic change in the rate of transcription of the *Hox-1.3* gene. Previous studies have shown that type IV collagen and B laminin gene transcription is increased in F9 cells upon addition of retinoic acid and dibutyryl adenosine 3′,5′-cyclic monophosphate (39). However, *maximal* transcriptional activation for these two genes does not occur until relatively late in the induction process (39). The increase in the rate of transcription for the *Hox-1.3* gene, 30-to 40-fold upon induction, is comparable to the increase we observe in the steady-state mRNA levels upon RAIC induction. Though this suggests that transcriptional rate changes play a major role in the accumulation of the major *Hox-1.3* mRNA, this does not rule out the possibility that posttranscriptional regulation

may play a role in the regulation of this gene product, as has been shown for c-*myc* (45–47) and p53 (45). It should be noted that we do not see an induction of the *Hox-1.3* transcripts when dibutyryl adenosine 3′,5′-cyclic monophosphate alone is added to the F9 cells, whereas we do see an induction when only retinoic acid is added to the cells (data not shown).

If one were to hypothesize a regulatory function for the *Hox-1.3* gene during development of the mouse, this protein might have different functions in different cell types given that it has been shown to be expressed in embryonic and adult tissue, in growing NIH 3T3 cells, and in differentiating F9 cells. Clearly, at this point in time, functional analysis of murine homeobox genes will require physical analysis of the proteins for possible DNA binding ability and genetic/gene transfer techniques that might illustrate function through inappropriate developmental expression of the gene or interference with expression by means of antisense RNA approaches.

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