Relationship between HOX2 homeobox gene expression and the human cytomegalovirus immediate early genes

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The human embryonal carcinoma cell line NT2/D1 is known to be non-permissive for human cytomegalovirus (HCMV) but becomes permissive after being induced to differentiate by retinoic acid (RA). Because homeobox genes have been reported to be specifically activated in the RA-differentiated NT2/D1 cells, we investigated the possible correlation between expression of homeobox (HOX) 2 genes and expression of the immediate early (IE) genes of HCMV both in NT2/D1 cells and in HCMV permissive human embryonic lung (HEL) cells. HCMV infection did not induce activation of the HOX2A, HOX2E and HOX2I genes in undifferentiated NT2/D1 cells nor affect their activation in the RA-differentiated NT2/D1 cells. By in situ hybridization using a HOX2A RNA probe, HOX2A transcript-positive cells appeared as clusters in RA-differentiated NT2/D1 cells. Viral antigen-positive cells detected by immunofluorescence using an antibody specific for the IE-1 antigen of HCMV appeared as clusters among the population of cells in which the HOX2A transcript was detected. The HOX2A gene only was expressed in HEL cells, however none of the HOX2 genes was expressed in non-permissive HeLa, Raji or mouse embryonic cells. These results suggest that activation of the HOX2A may be necessary for the expression of IE genes. HCMV infection markedly increased the expression of the HOX2E gene in HEL cells in the presence, but not in the absence, of cycloheximide. Ultraviolet-inactivated HCMV also displayed this effect. On the other hand, HCMV infection suppressed expression of the HOX2A gene to some degree at the early and late phases of infection in HEL cells. Activation of the HOX2E gene by HCMV might possibly have a role in virus-induced abnormal embryogenesis.

Human cytomegalovirus (HCMV), which displays a strict host cell type and species specificity, is a significant cause of congenital neonatal abnormalities (Weller, 1971; Stagno et al., 1982). Differential susceptibility to HCMV infection in certain cells in the embryo or foetus may cause abnormal embryogenesis or organogenesis, resulting in congenital abnormalities. The cellular and molecular mechanism underlying abnormal embryogenesis caused by HCMV is unknown.

Andrews et al. (1984) showed that the cloned human embryonal carcinoma cell line NT2/D1 can be induced to differentiate into various cell types, including neurons, by exposure to retinoic acid (RA). Gönçöl et al. (1984, 1985) reported that the undifferentiated NT2/D1 cell line is not permissive for HCMV replication but some of the cells become permissive when the line is induced to differentiate with RA. Differentiation of NT2/D1 cells by RA leads to a heterogeneous population of cells, including neurons which express cell surface tetanus toxin receptors (Andrews, 1984), all three neurofilament proteins (Lee & Andrews, 1985) and the S100β protein (Tsutsui et al., 1987). Moreover, Mavilio et al. (1988) reported that RA specifically induces the expression of a number of homeobox (HOX) genes in NT2/D1 cells. The HOX genes appear to regulate embryogenesis spatially and temporally not only in Drosophila but also in vertebrates (Gehring, 1987; Holland & Hogan, 1988). Recently, Simeone et al. (1990) reported that clustered HOX2 genes are sequentially activated in embryonal carcinoma cells in a 3' to 5' direction. It seems possible that some factors which are associated with cellular differentiation in embryogenesis may also regulate viral gene expression. Following infection of susceptible cells with HCMV, expression of the viral genome proceeds in three main phases: immediate early (IE), early and late (Stinski et al., 1980; DeMarchi, 1981; McDonough & Spector, 1983). The expression of IE genes is thought to be regulated by cellular factors (Nelson et al., 1990). In the present study, we show evidence of a correlation between expression of the HOX2A gene and transcription of the IE genes of HCMV, and of activation of the HOX2E gene by HCMV.

HCMV strain AD169 was obtained from Dr Y. Nishiyama, Nagoya University School of Medicine,
Japan. Virus stocks were prepared in human embryonic lung (HEL) cells and grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal calf serum (FCS), penicillin (100 units/ml) and streptomycin (50 μg/ml) (Tsutsui & Nogami-Satake, 1990). Virus was quantified by the plaque assay method of Wentworth & French (1970). Virus stock was 1 × 10^6 p.f.u./ml. The human teratocarcinoma cell line NT2/D1, cloned from the embryonal carcinoma cell line Tera-2 (Andrews et al., 1984), was cultured as described by Andrews et al. (1984). The cells were induced to differentiate by seeding at densities of 5 × 10^5 per 9 cm dishes (Falcon) in DMEM containing 10^-5 m-all-trans-RA (Eastman Kodak). Other cell types included the HeLa-S3 line derived from human uterus cancer, the Raji line derived from tumour tissue of a patient with Burkitt's lymphoma and mouse whole embryonic (MWE) cells prepared from mouse embryos. All the cells were cultured in DMEM containing 10% FCS, except for the Raji cells which were grown in suspension in RPMI 1640 medium containing 10% FCS. HOX2 DNA probes were kindly provided by Dr E. Boncinelli, International Institute of Genetics and Biophysics, CNR, Naples, Italy. Among the HOX2 clusters, we used three HOX2 probes from different positions; HOX2E (5' end of the cluster), HOX2A (middle of the cluster), HOX2I (3' end of the cluster) (Simeone et al., 1990). The XbaI-E fragment of HCMV (Towne strain) (Stinski et al., 1983) was provided by Dr Stinski, University of Iowa, Iowa City, U.S.A.

Total cell RNA was extracted from frozen samples by guanidium–caesium chloride centrifugation. Poly(A)+ RNA was selected by passage twice through an oligo(dT)-cellulose column (Pharmacia LKB) (Takahashi et al., 1985). Heat-denatured, 4 μg aliquots of poly(A)+ RNA in MOPS buffer (0-2 M-morpholino-propanesulphonic acid pH 7-0) containing 50% formamide and 6-6% formaldehyde was subjected to electrophoresis in a 1-0% denaturing agarose gel in MOPS buffer containing 2-2 M-formaldehyde. The RNAs were transferred to GeneScreen Plus membranes (New England Nuclear), then fixed by baking for 2 h at 80 °C under vacuum. The resulting membranes were hybridized with DNA probes radiolabelled with [α-32P]dCTP (ICN Radiochemicals) by Multiprime labelling system (Amersham) at 42 °C overnight in a hybridization solution containing 50% formamide, 5 × Denhardt’s solution, 10% dextran sulphate, 50 mM-Tris–HCl pH 7-5, 1 M-NaCl, 0-5% SDS, 100 μg denatured single-stranded salmon sperm DNA per ml and 100 μg yeast tRNA per ml. The membranes were washed with 2 × SSC twice at room temperature for 5 min, 2 × SSC–10% SDS twice at 60 °C for 30 min and 0-1 × SSC twice at room temperature for 30 min. Hybridization was detected by autoradiography using RXO-H film (Fuji) with HR-16 intensifying screen (Fuji Medical) at −80 °C.

RNA probes for in situ hybridization were prepared from the HindIII–BamHI fragment of HOX2A (1-1 kb) cloned in a plasmid vector, pGEM-4Z (Promega). Labelled RNA probes were synthesized by in vitro transcription (Melton et al., 1984) from the template DNA using digoxigenin-labelled uridine-triphosphate (Boehringer Mannheim). The antisense probe was distinguished from the sense probe by RNA dot blot hybridization using mRNA from differentiated NT2/D1 cells. The probes were degraded to 100 bases average length (Cox et al., 1984). NT2/D1 cells were plated on chamber slides (Nunc) with or without RA (1 × 10^-5 M). Twenty-four hours later, cells were either mock-infected or exposed to HCMV for 1 h at an m.o.i. of 5, then cultured in the growth medium with or without RA for appropriate times until day 6. Cells on slides were fixed in 4% paraformaldehyde in 0-1 M-phosphate buffer pH 7-0 at room temperature for 1 h and kept at 4 °C until use. In situ hybridization was performed as described (Wilkinson et al., 1987). After prehybridization at room temperature for 2 h, hybridization was carried out overnight at 42 °C. After incubation with antidigoxigenin antibody conjugated to alkaline phosphatase, the slides were visualized according to the manufacturer’s instruction (Boehringer Mannheim). After in situ hybridization, the slides were subjected to immunofluorescence using the monoclonal antibody E3, which is specific for the 72K IE protein of HCMV (provided by L. C. Goldstein, Genetic System Corporation, Seattle, U.S.A.) (Goldstein et al., 1982) as described previously (Tsutsui & Nogami-Satake, 1990).

Northern blot analysis of expression of the HOX2 genes and the IE genes of HCMV were performed in mock-infected and HCMV-infected NT2/D1 cells which were either undifferentiated or induced to differentiate by RA (Fig. 1). We confirmed the previous reports (Mavilio et al., 1988; Simeone et al., 1990) that HOX2 genes were activated when undifferentiated NT2/D1 cells were stimulated to differentiate by adding RA in the culture medium (Fig. 1a, b and c; lanes 1 and 2). Expression of the IE genes of HCMV occurred in only the RA-differentiated NT2/D1 cells (Fig. 1d). The viral infection did not alter the transcription of the HOX2 genes in RA-differentiated (Fig. 1a, b and c; compare lanes 2 and 4) nor undifferentiated NT2/D1 cells (Fig. 1a, b and c; compare lanes 1 and 3).

Expression of HOX2A, 2E and 2I was also examined in cells other than the NT2/D1 cell line. Only HOX2A was expressed in HEL cells which are permissive for HCMV (Fig. 2a, lane 2), whereas the gene was not expressed in non-permissive cells (HeLa, Raji and MWE cells; Fig. 2a, lanes 3, 4, 5, respectively). On the other
Fig. 1. Northern blot analysis of the HOX2 genes and the IE genes of HCMV in mock-infected and HCMV-infected NT2/D1 cells. Poly(A)⁺ RNAs (4 µg per lane) were hybridized with DNA probes (a) HOX2A, (b) HOX2E, (c) HOX2I and (d) XbaI-E fragment of HCMV. (a to d) Lanes 1, undifferentiated NT2/D1 cells after 7 days of culture; 2, NT2/D1 cells after 7 days of culture with RA; 3, undifferentiated NT2/D1 cells infected with HCMV 1 day after plating and cultured for 6 days; 4, NT2/D1 cells, plated with RA and infected with HCMV 1 day after and cultured for 6 days with RA. Open and black triangles indicate the mobility of the 28S and 18S rRNA, respectively. Profiles of agarose gels stained with ethidium bromide (EtBr) before blotting are presented to compare total RNA per lane.

Fig. 2. Expression of the HOX2 genes in different cell types. Poly(A)⁺ RNAs (4 µg per lane) were hybridized with (a) HOX2A, (b) HOX2E and (c) HOX2I DNA probes. (a to c) Lanes 1, differentiated NT2/D1 cells, cultured for 7 days with RA; 2, HEL cells; 3, HeLa-S3 cells; 4, Raji cells; 5, MWE cells. Open and black triangles indicate the mobility of 28S and 18S rRNA, respectively. Profiles of agarose gels stained with EtBr before blotting are presented to compare total RNA per lane.
Fig. 3. Comparison of localization of HOX2A mRNA detected by *in situ* hybridization and IE1 antigen detected by immunofluorescence in HCMV-infected NT2/D1 cells which were induced to differentiate by RA. NT2/D1 cells were plated on chamber slides with or without RA, infected with HCMV 1 day after plating and cultured for 3 days (a, b) or 6 days (c, d) with RA. *In situ* hybridization was performed using digoxigenin-labelled HOX2A antisense RNA probe, then the cells were incubated with antidigoxigenin antibody conjugated with alkaline phosphatase. After visualization, the same slides were subjected to immunofluorescence using monoclonal antibody E3, specific for IE1 antigen. (a and c) A cluster of cells, surrounded by black arrowheads, expressing HOX2A mRNA in HCMV-infected RA-differentiated NT2/D1 cells cultured for (a) 3 days and (c) 6 days. (b and d) The same field as in (a) and (c) is shown by immunofluorescence. (e) *In situ* hybridization to undifferentiated NT2/D1 cells using HOX2A antisense RNA probe. (f) *In situ* hybridization to the differentiated NT2/D1 cells cultured for 6 days with RA, using a HOX2A sense riboprobe. Cells indicated by white arrowheads in (a) and (c) correspond to those indicated by arrows in (b) and (d).

hand, HOX2E and 21 genes were not expressed either in permissive HEL cells or in the non-permissive cells (Fig. 2b and c).

To examine the possible correlation between activation of HOX2A expression and expression of the HCMV IE gene at the single cell level, the double staining method combining *in situ* hybridization with immunofluorescence was performed in HCMV-infected RA-differentiated NT2/D1 cells. By *in situ* hybridization using the HOX2A antisense RNA probe, the HOX2A transcript-positive cells appeared as clusters in the NT2/D1 cells treated with RA (Fig. 3 a and c; borders of clusters identified by black arrowheads); the intensity of hybridization varied between individual cells in the cluster. Although immunofluorescence staining for HCMV IE1 was weak after *in situ* hybridization, viral antigen-positive cells were indeed found (Fig. 3b and d) within the cluster of HOX2A transcript-positive cells (Fig. 3a and c). These positive cells tended to be rounded and to show relatively low levels of HOX2A transcript (Fig. 3a and c; white arrowheads correspond to those in Fig. 3b and d). The viral antigen-positive cells were always among the HOX2A transcript-positive cell population or associated with them, while the converse was not observed. No nuclear fluorescence was detected in the uninfected RA-treated NT2/D1 cells (not shown). Therefore, the viral antigen-positive cell population seemed to be a part of the cell population that expresses HOX2A mRNA. As controls, undifferentiated NT2/D1 cells contained few HOX2A transcript-positive cells (Fig. 3e), and no positive reaction was observed in the RA-treated NT2/D1 cells hybridized with the HOX2A sense RNA probe (Fig. 3f).

To examine the effect of HCMV infection on the expression of HOX2 genes in permissive cells, Northern blot analysis was performed using mRNA from HEL cells in the different phases of HCMV infection. To study the IE phase, cells were treated with cycloheximide (CH) (50 μg/ml) before, during and for 15 h after infection with HCMV at an m.o.i. of 3. For the early phase, cells were incubated with phosphonoacetic acid (PAA) (100 μg/ml) for 15 h after infection, whereas for the late phase, cells were incubated for 48 h in the absence of either inhibitor. Expression of HOX2A in the early and late phases (Fig. 4a, lanes 5 and 6, respectively) was slightly suppressed when compared with that of uninfected cells (Fig. 4a, lanes 1 and 4). When cells were treated with CH to achieve IE conditions, the expression...
Fig. 4. Expression of the HOX2 genes in HEL cells at different infection phases of HCMV. Poly(A)+ RNAs (4 μg per lane) were hybridized with (a) HOX2A, (b) HOX2E and (c) HOX2I DNA probes. (a) to (c) Lanes 1, uninfected cells; 2, uninfected cells in IE condition where cells were treated before, during and for 15 h after mock-infection with CH (50 μg/ml); 3, infected cells in IE condition; 4, uninfected cells in early condition where cells were treated with PAA (100 μg/ml) for 15 h; 5, infected cells in early condition; 6, infected cells in late condition where cells were cultured for 48 h after infection. Open and black triangles indicate the mobility of 28S and 18S rRNA, respectively. Profiles of agarose gels stained with EtBr before blotting are presented to compare total RNA per lane.

To examine further the activation of HOX2E by HCMV infection in IE-conditioned cells, u.v.-inactivated HCMV was prepared as described by Nishiyama & Rapp (1980). HCMV stocks were inactivated by treatment with two doses of u.v. light (254 nm); low u.v.-irradiated HCMV, 4 J/m².s for 2 min (survival was 4-5% by plaque assay) and high u.v.-irradiated HCMV, 4 J/m².s for 5 min (survival was less than 0.1%). Ultraviolet-inactivated or intact HCMV were adsorbed at the same concentration of virus (m.o.i. of 5 for intact virus) either on CH-treated or untreated HEL cells and incubated for 15 h with or without CH. The IE-transcripts were not detected in the cells infected with high u.v.-irradiated HCMV (Fig. 5b, lane 3), although they were detected slightly in the cells infected with low u.v.-irradiated HCMV (Fig. 5b, lane 2). In the presence of CH, activation of HOX2E was enhanced not just by intact HCMV (Fig. 5a, lane 4) but even by u.v.-inactivated virus preparations (Fig. 5a, lanes 2 and 3). In the absence of CH, no such activation was observed either by u.v.-inactivated or intact viruses (Fig. 5a, lanes 5 and 6).

The human embryonal carcinoma cell line, NT2/D1, is not permissive for HCMV replication but some of the cells become permissive when induced to differentiate with RA (Gönczöl et al., 1984, 1985), expression of some HOX2 genes induced in the RA-differentiated NT2/D1 cells (Mavilio et al., 1988; Simeone et al., 1990). In this study, we showed that only HOX2A but not HOX2E and HOX2I was expressed in permissive HEL cells and none of these genes was expressed in non-permissive HeLa, Raji or mouse embryonic cells. Furthermore, viral antigen (IE1)-positive cells always appeared in clusters of HOX2A transcript-positive cells detected by in situ hybridization. These facts suggest that expression of the HOX2A genes may be necessary for undifferentiated NT2/D1 cells to acquire susceptibility to HCMV infection. The HOX2A gene was reported to be located in the middle of the HOX2 gene cluster (Simeone et al., 1990). During differentiation of NT2/D1 cells, the order of HOX2A was markedly suppressed (Fig. 4a, lanes 2 and 3). In contrast, expression of HOX2E was markedly activated in the HCMV-infected IE-conditioned cells (Fig. 4b, lane 3); a much smaller amount of HOX2E expression was also induced in uninfected IE-conditioned cells (Fig. 4b, lane 2). Expression of the HOX2I gene was not observed in any phases of infection in HEL cells (Fig. 4c).
of cellular oncogenes fos, jun and myc. The activation of HOX2E was observed in the u.v.-irradiated HCMV-infected cells in the presence, but not in the absence, of CH. This suggests that suppression of de novo protein synthesis in the cells is essential for the activation of HOX2E by HCMV. In contrast, the expression of HOX2A in HEL cells appeared to be slightly suppressed when infected with HCMV. The present results therefore show that expression of the cellular HOX2 genes can be altered by HCMV infection. It is possible that such effects may underlie the capacity of HCMV infection to cause congenital abnormalities in vivo.

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References


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