

Induction of mcl1/EAT, Bcl-2 Related Gene, by Retinoic Acid or Heat Shock in the Human Embryonal Carcinoma Cells, NCR-G3

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ABSTRACT. NCR-G3 cells were established from a testicular embryonal carcinoma and were differentiated into multi-lineages including trophoblast cells by exposure to retinoic acid. The differentiated cells began to produce human chorionic gonadotropin (hCG), a trophoblast-specific hormone, which was regulated at the mRNA level. As we assumed that genes responsible for differentiation were differentially expressed at the early stage of retinoic acid-induced differentiation, we prepared a cDNA library from retinoic acid-treated NCR-G3 cells. This cDNA library was then screened for genes whose expression was induced during the differentiation of these cells. From about 5×10^4 clones screened, three independent sequences were isolated. Sequencing analysis revealed that clone 1002 codes for mcl1/EAT, which has a Bcl-2 homology domain. The expression of mcl1/EAT, the Bcl-2 related gene, was increased at an early stage of the retinoic acid-induced differentiation and preceded the up-regulation of cytokeratin and hCG genes after retinoic acid treatment. Furthermore, mcl1/EAT was also up-regulated by heat shock, which has recently been shown to induce the cells to differentiate.

A series of cellular events — growth, differentiation and cell death — occurs in the early embryogenesis of a human (19). A human being originates from a single fertilized egg and yet is composed of phenotypically different cells. It is a difficult task to analyze all the different cell lineages during the process of embryogenesis, especially in mammals. In order to conduct research in embryogenesis, despite their shortcomings, the use of animals such as sea urchins, nematodes, *Xenopus*, chickens and mice as model systems is necessary (9). In the determination and analysis of cell lineage, animals of simple structure or with a small number of cells such as nematodes are often used. For in vitro analysis of mammalian development, teratocarcinoma cells, especially pluripotent embryonal carcinoma (EC) cells, offer an opportune system to study the mechanism of early embryonic differentiation as well as malignant growth (18, 28, 29). In the murine system, EC cells or more recently, embryonic stem (ES) cells obtained directly from early-stage embryos have been successfully applied to the study of early events in embryogenesis (16, 18). However, in the human, germ cell tumors provide

the only material to study the mechanism of differentiation in early embryonic development (1, 10).

To determine the genes responsible for early embryogenesis of mammals, three approaches are available. The first strategy is to identify the molecules, especially transcription factors, which regulate the marker genes for differentiation (22). In this sense, major histocomplex genes and keratins are highly expressed at the eight-cell stage and are good markers for very early development. A specific hormone, human chorionic gonadotropin (hCG) is used to determine differentiation into trophoblast lineage (10). By a series of transfections into cultured cells and by generating transgenic mice, essential regulatory elements and their cognate proteins are identified (21). The second strategy is to utilize in vitro differentiation of EC cells or ES cells. Upon exposure to inducers, EC or ES cells differentiate into endodermal or trophoblastic lineage. Subtractive hybridization, differential display, and differential hybridization methods are employed to isolate up-regulated or down-regulated genes at the early step of in vitro differentiation. The third strategy is to generate knock-out mice or analyze mutant mice if the candidate genes are already known or predicted (3). Many events of development and differentiation have been elucidated at the mo-

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lecular level by these approaches.

The aim of this study was to isolate the essential genes in retinoic acid (RA)-induced differentiation of human EC cells. We isolated three clones, which were increased by RA. mcl1/EAT, one of the isolated clones, is expressed by RA or heat shock in the early stage of differentiation.

MATERIALS AND METHODS

Cell Culture. A 33-year-old man was admitted to Keio University Hospital, Tokyo, Japan, in February 1988, because of a testicular tumor. Orchiectomy was performed in March. The tumor histology was that of mixed embryonal carcinoma. The resected tumor was used to establish a cell line. The patient died of multiple organ failure in February 1990 (autopsy No.14137). An immortalized cloned cell line was obtained from the tumor described above and was designated NCR-G3 cells (10). The cell line was cultured with a 1:1 mixture of Dulbecco's modified Eagle medium and Ham's F12 medium (GIBCO, Grand Island, N.Y.) supplemented with 10% fetal calf serum, Insulin-Transferrin-Sodium Selenite media supplement (insulin, transferrin, 5 mg each; sodium selenite, 5 ng/ml), D-glucose (3.5 g/l), penicillin, and streptomycin. NCR-G3 cells were treated with either 2×10^{-5} mol of all-trans-RA (Sigma Chemical Co., St Louis, Mo.) or 0.5 nM of 12-*o*-tetradecanoylphorbol 13-acetate (TPA).

RNA blot analysis. RNA was prepared from the cultured cells and tissues as previously described (32). Tissues from the placenta, kidney, adrenal gland and testis were obtained from surgical samples. RNAs from the brain and liver were purchased from Clontech (Palo Alto, Calif.). The RNA was then electrophoresed in a 1.0% agarose gel, transferred to a nylon filter (NEN Research Products, Boston, Mass.), and hybridized with cDNA inserts labeled with ^{32}P -dCTP by the random-primer method (7) at 65°C for 14–16 hours in a buffer containing $5 \times \text{SSPE}$ ($1 \times \text{SSPE}$ is 0.18 mole of NaCl, 10 mmol of $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ [pH 7.4], 1 mmol of EDTA), $5 \times \text{Denhardt's}$ solution ($1 \times \text{Denhardt's}$ solution is 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin), 0.02% poly(A), and 1% sodium dodecyl sulfate (SDS). The blots were washed with $2 \times \text{SSC}$ containing 1% SDS at room temperature and 65°C. Final washings were with $0.1 \times \text{SSC}$ containing 0.1% SDS at 65°C. The blots were exposed to X-ray film at -80°C using an intensifying screen. The hCG- β probe was a 0.6-kb *Hind*III fragment from pchCG- β (8).

Measurement of hCG. The medium was harvested from RA-treated or untreated NCR-G3 cells which were grown in medium supplemented with 10% fetal calf serum and antibiotics. hCG in the medium was measured by time-resolved fluoroimmunoassay (23). The antibody used in the immunoassay reacts with hCG but not with other hormones such as luteinizing hormone or follicle-stimulating hormone.

Immunohistochemistry. Rabbit antibody against hCG (Mochida Pharmaceutical Co., Ltd., Tokyo, Japan) was used

as the primary antibody for the indirect peroxidase method. Horseradish peroxidase-labeled anti-rabbit IgG (Dako Laboratories, Copenhagen, Denmark) was used at a 1:50 dilution. The working solution for the peroxidase reaction contained 30 mg of 3,3'-diaminobenzidine tetrahydrochloride (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and 65 mg of sodium azide per dl and 0.003% hydrogen peroxide in 0.05 M Tris-HCl buffer, pH 7.6. As a negative control, the primary antiserum was replaced by non-immune rabbit serum.

Preparation of Subtracted cDNA Probe. The protocol of subtraction was followed according to the method of Davis *et al.* (5). Total RNA was isolated from NCR-G3 cells after 8-hour exposure to RA. Poly(A)⁺ RNA was isolated from total RNA with Oligotex dT-30 (Takara Shuzo Co. Ltd., Kyoto, Japan). ^{32}P -dCTP-labeled cDNAs from poly(A)⁺ RNA of RA-treated NCR-G3 cells were synthesized with an oligo(dT) primer by Moloney murine leukemia virus reverse transcriptase (SuperscriptTM, Bethesda Research Laboratories, Inc., Gaithersburg, Md.). Two successive cycles of subtractive hybridization were performed on the ^{32}P -labeled cDNA with 35 to 50 μg of poly(A)⁺ RNA of untreated NCR-G3 cells. Specific activity of these probes was about 6×10^5 cpm/ μg . Reactions of the first and second subtractive hybridization were achieved with R_{OT} values of approximately 7,000 and 4,000 mol of nucleotide/sec/liter, respectively. Single-strand material was selected over hydroxyapatite equilibrated in 0.10 M $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ (pH 7.0), 0.1% SDS at 60°C in a water-jacketed column (Bio-Rad). The percentage of material remaining after the two subtraction cycles was found to be 1.9%. This single-strand material was used as the RA-treated NCR-G3 probe for the first screening. The exogenously added poly(A)⁺ RNA of the neo gene transcribed from pSP64Aneo was concentrated about 4- to 7.5- and 2-fold by the first and the second subtractive hybridization, respectively.

Library Screening. The RA-treated NCR-G3 cDNA library was prepared by a ZAP-cDNA synthesis kit (Stratagene, La Jolla, Calif.). Plaque hybridization was performed with replicate nylon filters (Hybond-N, Amersham Japan Ltd. Tokyo) from plates containing 5×10^4 cDNA clones. NCR-G3 probes or subtracted probes (1.1×10^6 cpm/ml) were used. The hybridization was carried out for 16 hours at 65°C in $5 \times \text{SSPE}$, $5 \times \text{Denhardt's}$ solution, 0.01% poly(A), 1% SDS. The blots were washed twice in $1 \times \text{SSPE}$, 1% SDS at room temperature for 10 minutes, and twice in $1 \times \text{SSPE}$, 1% SDS at 65°C for 15 minutes each. The autoradiogram was exposed for seven days at -80°C using an intensifying screen.

A total of 577 cDNA clones were initially picked and subcloned. The isolated plaques were individually gridded and screened on duplicate nylon filters with either ^{32}P -labeled RA-treated NCR-G3 cDNA or untreated NCR-G3 cDNA. After the second screening, 67 cDNA clones were picked. Cross hybridization tests were performed on the inserts to avoid redundant clones. These cDNAs were amplified by the polymerase chain reaction with a thermal cycler (Cetus, Emeryville, Calif.) and used as a probe in RNA blot analysis.

Sequencing. pBluescript containing cDNAs from uni-ZAP XR vector was excised *in vivo* in order to determine the sequence of each clone. Sequencing was done according to the cycle-sequencing protocol (Applied Biosystems Inc., Foster City, Calif.) with automated DNA sequencing system 373A (Applied Biosystems). The cDNA sequences were analyzed for homology with sequences in the EMBL and GenBank DNA databases by using the Software Development Corp. (Tokyo) program.

Fluorescence *in situ* hybridization (FISH). FISH was performed as described elsewhere (20). In brief, metaphase chromosomes were prepared by thymidine synchronization, and bromodeoxyuridine release technique for delineation of replication G-band. The slides were denatured in 70% formamide/2×SSC (0.3 M NaCl; 0.03 M trisodium citrate) at 75°C for 2 min, immersed in 70% ethanol at −20°C, and dehydrated through an ethanol series. A 2.4 kb full length cDNA designated as mcl1/EAT was used as a probe. The probe DNA (1 μg) was labeled with biotin-16-dUTP (Boehringer Mannheim) by nick-translation reaction. The hybridization was performed in a solution containing 50% formamide, 10% dextran sulfate, 2×SSC, and DNAs dissolved to concentrations as followings: 50 ng/μl of biotinylated probe DNA, 0.5 μg/μl of sonicated salmon sperm DNA, and 0.5 μg/μl of *Escherichia coli* tRNA. The hybridization signals were detected with FITC-avidin (Boehringer Mannheim) and biotinylated anti-avidin (Vector) as described (24).

RESULTS

To isolate the genes responsible for differentiation, it is important to examine the differentiation pathway in NCR-G3 cells in detail. First, we determined the changes in expression of a trophoblastic giant cell-specific hormone (hCG) and trophoblast-associated cytokeratins during the differentiation of a human EC cell line, NCR-G3 (Fig. 1). hCG is produced mainly in syncytiotrophoblasts of placental tissue and is one of the molecular markers of terminally differentiated trophoblast cells.

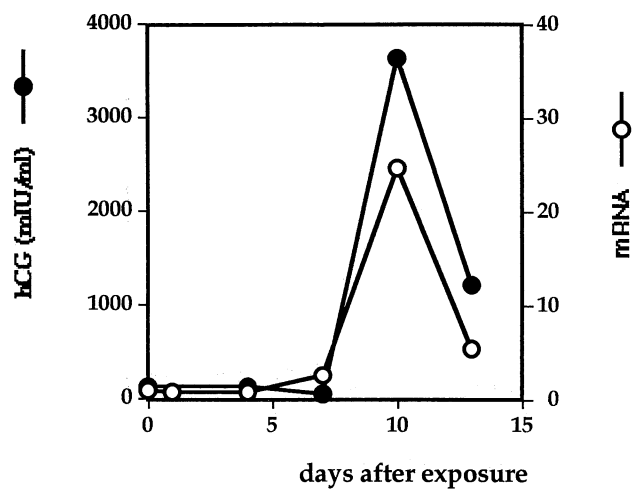
Cells were treated with RA for seven days (days 0–7) and then cultured without any treatment (days 8–13). NCR-G3 cells began to produce hCG on day 10 (Fig. 1A). Without exposure to RA, hCG production was not observed. We also measured α -fetoprotein (AFP) in the supernatant of the cell cultures since the original tumor in the patient produced AFP. No AFP production was detected in NCR-G3 cells during the RA-induced differentiation (data not shown).

To determine whether the production of hCG is regulated at the mRNA level or at the protein level, blot hybridization of total RNA from RA-treated NCR-G3 cells was carried out with the hCG- β probe. Expression of the hCG- β gene was below detectable levels or was extremely low during 7 days of exposure to RA (Figs. 1A and 1B). hCG mRNA was induced on day 10. The up-regulated expression of the hCG- β gene was transient since hCG- β mRNA decreased on day 13. The time kinetics of the hCG RNA level was almost the same as that of hCG protein production in the supernatant of the cells. hCG mRNA specifically decreased on day 13. This effect was not a result of general RNA degradation, as similar amounts of actin mRNA were observed in each lane (Fig. 1B, bottom panel).

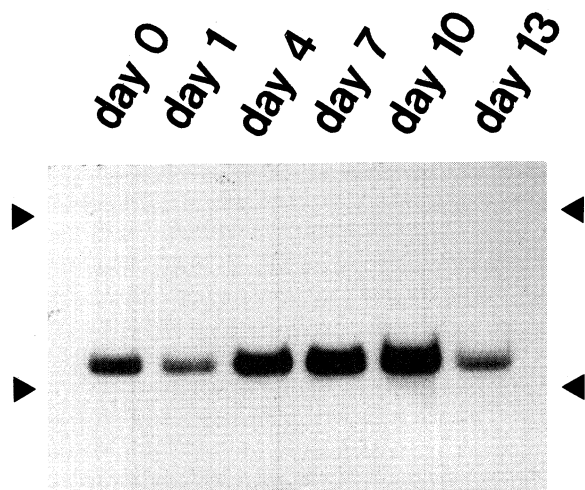
To study the relationship between expression of hCG and other markers for differentiation, blot hybridization was done with cytokeratin No. 8 probe using the same differentiation protocol as the blot for the hCG probe (Fig. 1C). The cytoskeletal protein encoded by the gene is reported to be a trophoblast- or epithelium-associated molecular marker. NCR-G3 cells expressed the gene constitutively. The cytokeratin mRNA was up-regulated significantly on day 4 or day 7 of exposure to RA. Cytokeratins No. 18 and No. 19 were induced by RA treatment in a similar manner (data not shown). During differentiation induced by RA, NCR-G3 showed a subtle alteration in morphology (Figs. 1D and 1E) even though the cytokeratins were differentially regulated. Multinucleated giant cells, which are positive for hCG, increased after RA treatment, implying that

Fig. 1. Production and expression of hCG in NCR-G3 cells exposed to RA.

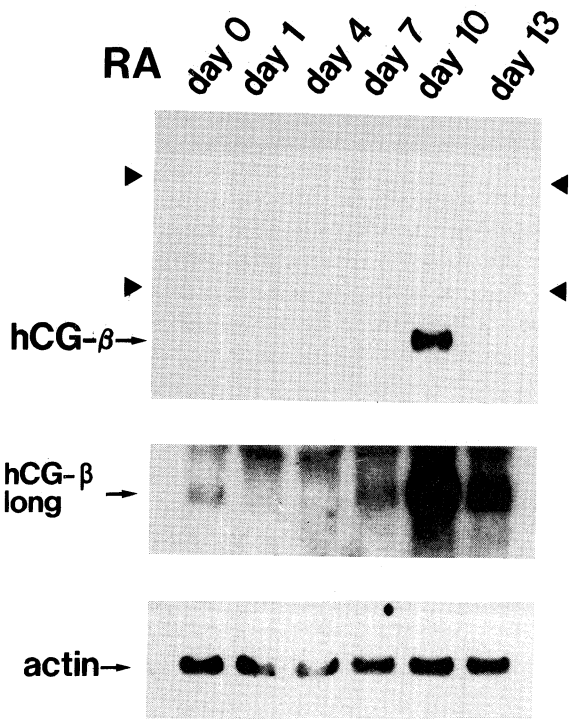
(A), Time-course of hCG production (closed circle) and hCG- β transcription (open circle) in NCR-G3 cells during differentiation. Cells were treated with 2×10^{-5} moles of RA for seven days (days 0–7). After the RA treatment, cells were cultured without any treatment. Supernatants of cultured cells treated with RA were collected on the indicated days for the determination of hCG production. The culture medium was changed 3 days before collection. Relative expression of hCG- β was determined densitometrically from autoradiograms of RNA blot analysis. The amount of hCG mRNA without treatment (day 0) was arbitrarily set as 1.0. (B), RNA was extracted from NCR-G3 cells on the indicated days. Ten micrograms of total RNA was electrophoresed in each lane. The blot was hybridized with a hCG- β probe (top and middle panels) first. The filters were exposed for 1 day (top panel) and 7 days (middle panel, hCG long) at −80°C, using an intensifying screen. The blot was then dehybridized with a solution of boiled 0.01% SDS and 0.01×SSC five times and rehybridized with the actin probe. The positions of the 28S and 18S rRNA are indicated by arrowheads. (C), RNA was extracted from NCR-G3 cells on the indicated days. Ten micrograms of total RNA was electrophoresed in each lane. The blot was hybridized with the cytokeratin 8 cDNA probe described in Materials and Methods. The positions of the 28S and 18S rRNA are indicated by arrowheads. The autoradiograms were exposed for 24 hours at −80°C using an intensifying screen. (D) and (E), Phase contrast photograph of undifferentiated and differentiated NCR-G3 cells. NCR-G3 cells grew in two forms, as both floating cell aggregates and flattened cells attached to the culture dish (D). Cells were treated with 2×10^{-5} moles of RA for seven days (days 0–7). After the RA treatment, cells were cultured without any treatment (E). On day 11 after treatment, multinucleated giant cells were identified and were positive for hCG by immunohistochemistry (inlet).



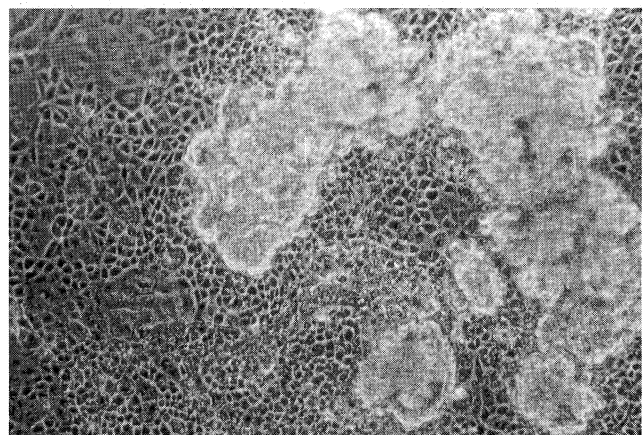
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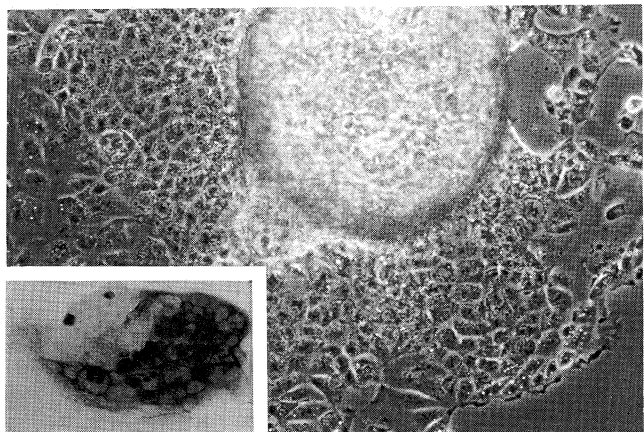
C



B



D



E

Fig. 1.

the cells differentiated into syncytiotrophoblastic giant cells (Fig. 1E, inset).

Isolation of cDNAs which are induced at an early stage of differentiation of NCR-G3 cells. Assuming that genes responsible for differentiation exhibit increased expression at an early stage of differentiation, by subtractive hybridization we cloned genes that are induced by 8-hour treatment with RA. Subtractive hybridization of NCR-G3, which was treated with RA for 8 hours, was conducted with excess poly(A)⁺ RNA of the undifferentiated NCR-G3 and this subtracted cDNA preparation was used as the probe for screening the cDNA library of NCR-G3 which was exposed to RA for 8 hours.

cDNA clones whose RNA was induced by RA were searched by differential hybridization procedure using probes of both subtracted cDNA described above and cDNA synthesized from untreated NCR-G3 cells. Plaques whose signals were stronger when the clones were probed by the subtracted cDNA were picked from the original plates. From 5×10^4 cDNA clones screened, 67 cDNA clones were isolated.

Expression of three independent cDNA clones, which are referred to as 1002, 0734 and 2403, was shown by RNA blot analysis to be up-regulated (Fig. 2A). Clones 1002 and 2403 represented RNAs of approximately 4.0 and 2.7 kb, respectively. Clone 0734 showed a range of RNAs although a discrete band of approximately 6.5 kb was observed. Clone 1002 seemed to be less abundant since longer exposure was necessary to visualize the discrete signal compared with clones 2403 and 0734 (see legend for Fig. 2).

Sequencing of the three selected cDNA clones and expression of mcl1/EAT gene. Clones 1002, 2403 and 0734 were sequenced and searched for homology by computer-aided comparison. 1002 was an unknown sequence; 2403 was found to be a 90-kD heat shock protein (hsp90) (25); 0734, Line-1, was found to be a member of the repetitive sequence family (11, 28). To study the time-kinetics of expression of each clone, at various times we isolated RNA of NCR-G3 cells treated with RA. The 1002 transcript began to be expressed after 8 hours of treatment (Fig. 2B) and reached the maximal level on day 2 (data not shown). Since 1002 is induced at an early stage of differentiation, we have named the clone EAT (early gene induced by all-trans RA in human EC cells). We have recently identified EAT as the reported gene mcl1 that is induced by TPA in the myeloid leukemic cells (15). Sequence analysis revealed that 1002 encodes the 3' untranslated region of the gene.

We have recently found that NCR-G3 cells are differentiated by heat shock as well as by RA (17). To determine whether mcl1/EAT regulation is accompanied by differentiation even by heat shock, we performed RNA blot analysis in cells after heat shock. mcl1/EAT was

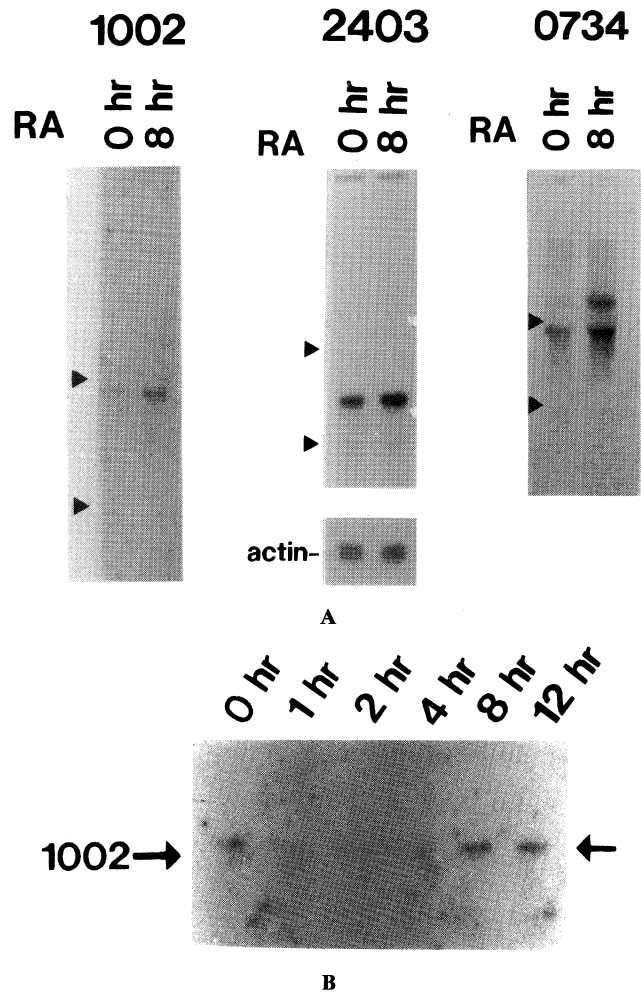


Fig. 2. RNA blot analysis by the probes of isolated cDNA clones. (A), NCR-G3 cells were treated with 2×10^{-5} moles of RA for 8 hours. RNA was extracted from RA-treated and untreated NCR-G3 cells. Five micrograms of total RNA was electrophoresed on each lane. The blot was hybridized with the 1002, 2403 and 0734 cDNA probes described in Materials and Methods. The autoradiograms were exposed for 7 days (1002), 2 days (2403), and 1 day (0734) at -80°C using an intensifying screen. (B), Time-kinetics of expression of clones 1002 (mcl1/EAT) in NCR-cells exposed to RA. NCR-G3 cells were treated with 2×10^{-5} moles of RA. The total RNA was extracted on the indicated hours after the addition of RA using cDNA probe 1002 (mcl1/EAT). Five micrograms of the total RNA was electrophoresed in each lane. The amount of total RNA was checked by 28S and 18S rRNA stained with methylene blue and the level of expression of the actin gene on each blot.

clearly induced 8 hours after heat shock (Fig. 3). Since mcl1/EAT was originally isolated as a gene which is increased by phorbol ester, TPA, we tested if mcl1/EAT is regulated by TPA. mcl1/EAT transcripts were not up-regulated 8 hours after treatment of 0.5 nM TPA.

Tissue-specific expression and chromosomal localization of mcl1/EAT. To determine the expression of the

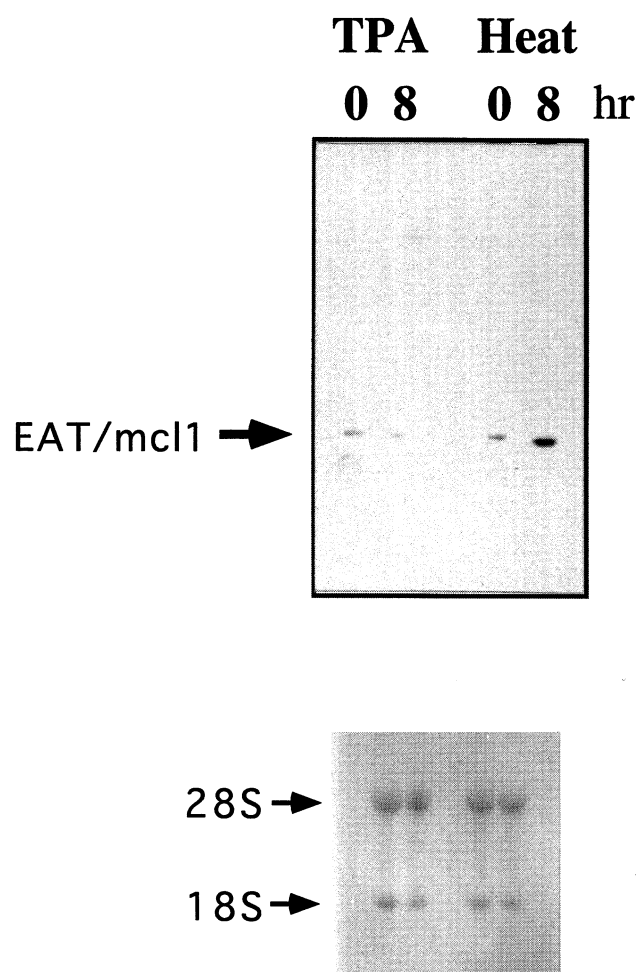


Fig. 3. mcl1/EAT expression in NCR-G3 cells exposed to phorbol ester and heat shock.

RNA was extracted from NCR-G3 cells 8 hours after 0.5 nM TPA or heat shock at 42°C. Ten micrograms of total RNA was electrophoresed in each lane. The blot was hybridized with the mcl1/EAT cDNA probe described in Materials and Methods. Almost exactly the same amounts of the 28S and 18S rRNAs of NCR-G3 cells were detected in both control and treated cultures (bottom). The autoradiograms were exposed for 16 hours at -80°C, using an intensifying screen.

gene in human tissues, blot hybridization analysis was done with mcl1/EAT, the untranslated sequence of the gene, under high stringency conditions (Fig. 4). Placenta exhibited high levels of mcl1/EAT transcripts. This is consistent with the results that mcl1/EAT was isolated as a clone which was up-regulated during the trophectoderm differentiation of EC cells. Levels of mcl1/EAT expression remain unchanged in the placenta during pregnancy (data not shown). Brain and adrenal gland contained significant amounts of mcl1/EAT mRNA, while kidney and testis had very low levels of the transcript.

Chromosomal localization of mcl1/EAT gene was

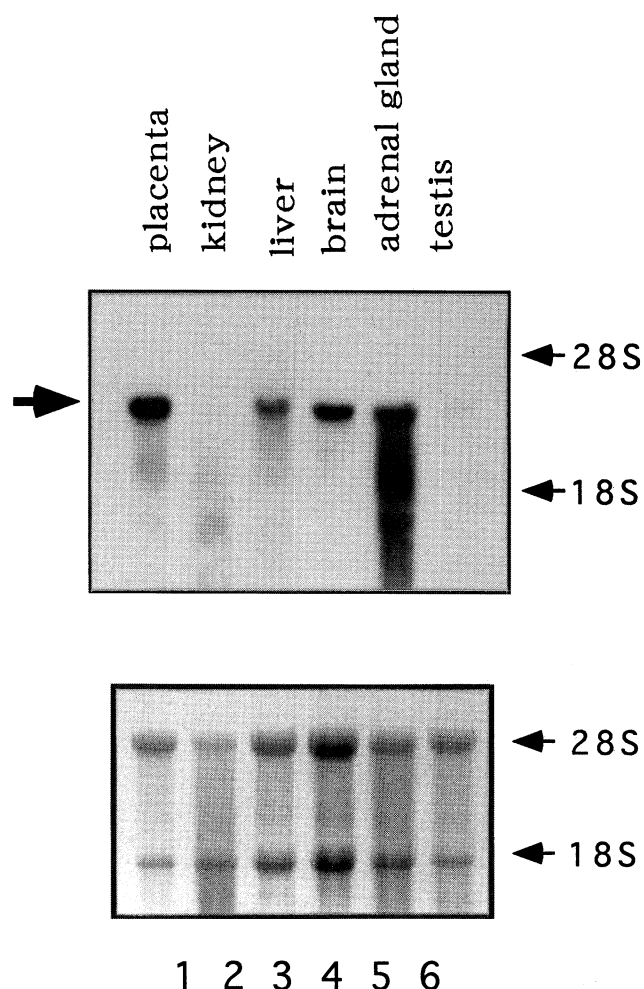


Fig. 4. Expression of mcl1/EAT gene in human tissues.

RNA was extracted from placenta, kidney, liver, brain, adrenal gland, and testis (from left to right, lanes 1-6). Ten micrograms of total RNA was electrophoresed in each lane. The blot was hybridized with the mcl1/EAT cDNA probe described in Materials and Methods. A general RNA degradation was not observed, since the 28S and 18S rRNAs were recovered from all the tissues (bottom).

determined by FISH (Fig. 5). A total of 50 metaphase cells were examined. Of these, 9 cells exhibited twin-spot signals on both homologous chromosomes 1 at q21, and another 14 cells showed twin-spot signals on one chromosome 1q21. Such specific accumulation of signals could not be detected on any other chromosomes. These results indicate that mcl1/EAT gene locates on chromosome 1 at q21. The chromosomal mapping of mcl1/EAT in this paper and others is interesting in the light of the finding that control of human cellular senescence is on chromosome 1q (4, 12, 30). Fas ligand, another cell death or apoptosis-related gene, is also located on chromosome 1q near mcl1/EAT gene (31).

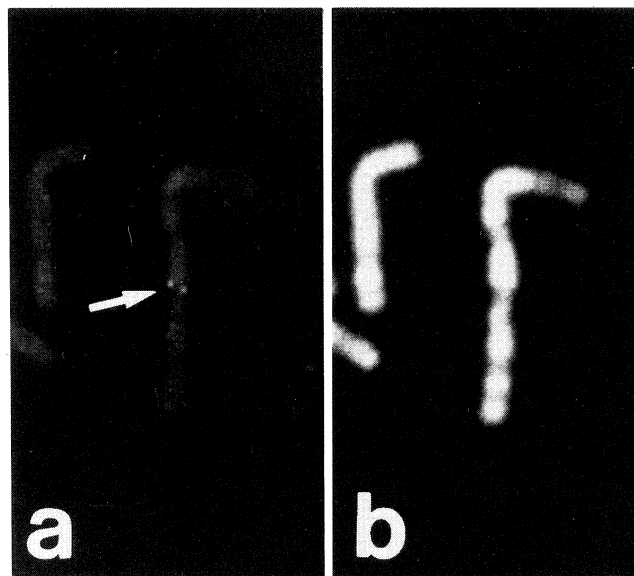


Fig. 5. Chromosomal assignment of the human mcl1/EAT gene by FISH.

(a), Twin-spot signals specific for mcl1/EAT (arrow) were detected on both sister chromatids of the long arm of chromosome 1. (b), G-band pattern of the same chromosomes were delineated through UV-filter, showing that the human EAT gene locates on 1q21.

DISCUSSION

mcl1/EAT gene, Bcl-2 related gene, is up-regulated at an early stage of the differentiation of human EC cells, NCR-G3. Human EC cell lines have been used as model systems for the study of early human embryogenesis, since these tumors are able to differentiate into a variety of cell types and are considered human counterparts of murine embryonal carcinoma cells (1, 2, 29). The differentiation capacity of these human EC cells as well as other cells (6, 13, 32) provides a means of isolating molecules which have a role in transcriptional regulation during the RA-dependent morphogenetic process. In this study, we tried to isolate the genes responsible for the differentiation of human EC cells, NCR-G3, which were generated from a mixed embryonal carcinoma of human testis.

The molecule that triggers the differentiation program is thought to be rare and transiently expressed in the early stage of differentiation. From this point of view, mcl1/EAT, one of the isolated genes, is of interest since its expression is very low and shows temporal regulation. mcl1/EAT has been reported elsewhere to be isolated from the myeloid leukemia cell line during phorbol ester-induced differentiation along the monocyte/macrophage pathway and to have a bcl-2 homology domain (15). The Bcl-2 family genes have been

shown to be correlated with apoptotic death (14, 26). These genes seem to have opposite effects on cell death; bcl-2 and bcl-xL inhibit apoptosis while Bax and bcl-xs accelerate apoptosis (27). Upon RA exposure, NCR-G3 cells undergo apoptosis with condensation of chromatin and degradation of genome DNA. While we still do not know whether mcl1/EAT is involved in the induction or inhibition of cell death, it is quite interesting that the bcl-2 related gene increased during the early stage of human EC cell differentiation.

mcl1/EAT gene is induced by RA or heat shock. In NCR-G3 cells, retinoic acid or heat increase mcl1/EAT expression. Interestingly, upon RA or heat exposure, human NCR-G3 has been shown to differentiate into the trophoblast lineage (10, 17). Thus, mcl1/EAT induction is correlated with the event of differentiation. Also, the expression of mcl1/EAT in the human placenta is consistent with the up-regulation of the mcl1/EAT transcript during trophoblast differentiation of NCR-G3 cells. In the placenta, the EAT gene is specifically expressed in trophoblastic cells as demonstrated by in situ hybridization analysis (A. Suzuki, A. Umezawa and J. Hata, unpublished observation). Since the transfection efficiency of the gene in NCR-G3 cells is extremely low, an alternative approach is required to determine a comprehensive role of the gene in the development. Our goals in the future are: a) targeting both alleles of the gene in NCR-G3 or other cells to determine whether the cells can differentiate without the mcl1/EAT products; likewise b) targeting of the gene in mice to determine whether mcl1/EAT products are essential for the organogenesis.

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