Korean J Biol Sci 1: 143-150, 1997

Analyses of Transcription Factor CP2 Expression during Development and Differentiation

Ji Hyung Chae, Eun-Jung Oh[1], and Chul Geun Kim*

Department of Biology, College of Natural Sciences, Hanyang University, Seoul 133-791, Korea;
[1]Department of Molecular Biology, Graduate School of Chonbuk National University, Chonju 560-756, Korea

Key Words:
Transcription factor CP2
Development
Differentiation
RT-PCR
In situ hybridization
α-Globin gene
Expression profile

Transcription factor CP2 was identified initially to bind the promoter region of the murine α-globin gene and its activity was shown to increase 2 to 3 fold during the induced differentiation of murine erythroleukemia (MEL) cells. To get further insight into the role of CP2 during development and differentiation, steady-state levels of CP2 message were monitored by using reverse transcriptase (RT)-PCR and *in situ* hybridization assays in the cultured MEL cells and differentiating embryonic stem (ES) cells *in vitro*, and in fetal and adult mouse tissues. The amount of CP2 messages increased 3 to 5 fold during induced differentiation of MEL cells, suggesting that the increment of CP2 activity during induced differentiation of MEL cells is originated from the increase of transcription initiation. On the other hand, CP2 expression is not restricted to the erythroid lineage cells; CP2 expressed ubiquitously from the undifferentiated ES cells to adult tissue cells. CP2 transcript was observed even in the undifferentiated ES cells and the level of expression increased from day 8 of the differentiating embryoid bodies. RT-PCR assay in the total RNAs prepared from several tissues of the adult mouse also showed ubiquitous expression profile, although the levels of expression were variable among tissues. When non-radioactive *in situ* hybridization assay was performed to the paraffin-sectioned whole body mouse embryos at days 11.5, 13.5, and 16.5 after fertilization, variable amounts of positive signals were also detected in different tissues.

CP2 is a transcription factor present in the nuclear extracts prepared from murine erythroleukemia (MEL) cells and a variety of other cell lines (Barnhart et al., 1988; Chodish et al., 1988; Kim et al., 1988). Additional characterization of the purified factor showed that CP2 recognizes a hyphenated DNA sequence element composed of 4 base motifs separated by a linker of 5 or 6 bases {CNRG-N(5-6)-CNR(G/C)} in the murine α-globin promoter (Kim et al., 1990; Lim et al., 1993). Even though any tissue tropism of CP2 expression or target genes of CP2 were not thoroughly characterized, similar CP2 binding sites were also identified in the upstream of the γ-fibrinogen, SV40 late and human immunodeficiency virus (HIV-1) promoters (Jones et al., 1988; Kim et al., 1990; Cullen, 1991; Swendeman et al., 1994). CP2, also known as LBP-1 by several groups, interacts with a CTGG element repeated three times in the -3 to +20 region of the HIV-1 LTR and mediates restriction of HIV-1 transcription at the level of elongation (Jones et al., 1988; Kato et al., 1991; Zhong et al., 1994; Parada et al., 1995).

* To whom correspondence should be addressed.
Tel: 82-2-290-0957, Fax: 82-2-296-5996

Our interest in CP2 originated from the initial finding that the murine α-globin promoter contains strong CP2 binding sites in a region that partially overlaps with the CP1 binding site (CCAAT box; also termed NF-Y binding site) (Dorn et al., 1987; Barnhart et al., 1988; Kim et al., 1988). Indeed, CP2, purified by using a DNA sequence affinity column based on the α-globin promoter sequence (Kim et al., 1988), was shown to be a potent activator of synthetic templates comprised of CP2 sites juxtaposed to the α-globin TATAA-box (Kim et al., 1990). Furthermore, among several factors that bind to the α-globin promoter, only CP2 showed increment of its transcriptional activity (2 to 3 fold) during the HMBA-induced terminal differentiation of MEL cells, suggesting that CP2 is the factor directly involved in the transcriptional activation of α-globin gene (Kim et al., 1990). It was also shown that overexpression of the cloned factor *in vivo* can activate the natural α-globin promoter template approximately 4 fold in a factor binding site dependent manner (Lim et al., 1993).

On the other hand, more recently Jane *et al.* (1995) have demonstrated that, while the transcription factor CP2 has been shown to bind to several cellular and viral promoters as a homodimer (Lim et al., 1992, 1993; Yoon et al., 1994), CP2 can form a

heterodimer with a 45 kD partner protein to form the stage selector protein complex (SSP) that binds to a proximal γ-globin promoter regulatory element, the stage selector element (SSE), which appears to be involved in the competitive silencing of the β-globin in the fetal stage of erythropoiesis (Jane et al., 1992, 1993: for general descriptions on globin gene switching and LCR, see Kim, 1993; Kim et al., 1992; Fiering et al., 1993; Orkin, 1995; Guy et al., 1996; Martin et al., 1996 and references therein). Similar SSP-like binding sites have been identified in the ε-globin promoter and regulatory elements of the β-globin locus control region (Gumucio et al., 1993). The importance of the SSP complex is further emphasized by the fact that the chicken stage selector protein, NF-E4, which is the avian homologue of CP2, has been implicated in the competitive silencing of the chicken ε-globin gene (Jane et al., 1995).

Even though CP2 might be very important in globin gene switching in erythroid cell lineages, both functional role and target genes in other tissues during development and differentiation, are not understood yet. In this study, to get more insight into tissue tropism in CP2 expression during normal development and differentiation, steady-state levels of CP2 message were monitored by using reverse transcriptase (RT)-PCR and *in situ* hybridization assays in cultured MEL cells and differentiating embryonic stem (ES) cells *in vitro*, and in fetal and adult mouse tissues.

Materials and Methods

Cell culture

The mouse ES cell line used in these experiments was 129/sv-derived CCE (Kim, 1996a, b). ES cells were adapted to grow in the presence of leukemia-inhibitory factor (LIF) without feeder cells (Wiles and Keller, 1991). Undifferentiated ES cells were maintained on gelatinated petri dishes (Costar) in Dulbecco's modified Eagle medium (DMEM, Gibco/BRL) supplemented with LIF, bovine calf serum (BCS) (15% final concentration, Hyclone), and monothioglycerol (1.5×10^{-4}M). LIF was obtained as a conditioned medium from a LIF-transformed CHO cell line (Nichols et al., 1990).

MEL (mouse erythroleukemia) DS19 cells were grown to densities of 1×10^6 to 2×10^6 cells per ml in the DMEM supplemented with 10% BCS as described previously (Cohen et al., 1986). Cells were induced in the presence of 5 mM hexamethylenebisacetamide (HMBA, Sigma).

In vitro differentiation of ES cells in suspension culture

Differentiation of ES cells was carried out essentially as described elsewhere (Kim, 1996a) with minor modifications to the original description (Zhuang et al., 1992). Confluent ES cell cultures were harvested by trypsinization and then resuspended at a concentration of 1×10^5 cells per ml in differentiation medium (DMEM supplemented with 10% fetal bovine serum). Ten milliliter aliquots were dispensed into 100 mm bacterial petri dishes (Baxter) to allow embryoid bodies (EBs) to form. The suspension was diluted 1:3 on day 3 and maintained in a humidified 5% CO_2 atmosphere as long as the experiment required; the medium was changed every 3 days.

RNA preparation and RT-PCR analysis

Total RNA was isolated from differentiating EBs and mouse tissue samples using RNAzol method (Chomczynsky and Sacchi, 1987). To get RNA from tissue samples, freshly excised tissue was dropped into liquid nitrogen and then frozen sample was ground to a powder in the mortar. After the powdered tissue was transferred into 1.5 ml microcentrifuge tube, the tissue homogenate was dissolved in RNAzol by repeated pipetting. In case of cultured cells, collected cells were directly dissolved in RNAzol by repeated pipetting. The quality of prepared total RNA samples was checked by electrophoresis on TBE-based native agarose gels using normal DNA loading buffer in the increased electric field (10 V/cm) (Park et al., 1996).

RT-PCR assay was performed essentially as described (Kim, 1996a). Briefly, 100 ng of each RNA sample was used for the RT reaction with random hexamers; a portion of the resulting cDNAs was amplified with a set of CP2 exon-specific primers for 28 cycles. As an internal control, a set of exon-specific primers in hypoxanthine-guanine phosphoribosyltransferase (HPRT) gene and mouse β-type globin genes was also used for each RT samples. The primer sets used in this study are as follows: CP2, 5'-GCA CTC GGC CAG CTG CCA GA-3' and 5'-TGG AGG GGG TGG TTC TGG CT-3'; HPRT, 5'-CAC AGG ACT AGA ACA CCT GC-3' and 5'-GCT GGT GAA AAG GAC CTC T-3'; β-globin (εY), 5'-AAC CCT CAT CAA TGG CCT GTG G-3' and 5'-TCA GTG GTA CTT GTG GGA CAG C-3'; β-globin (βH1), 5'-GGA AAC CCC CGG ATT AGA GC-3' and 5'-CAG CCT GCA CCT CTG GGG TG-3'; β-globin (β-maj), 5'-ATG GTG CAC CTG ACT GAT GCT G-3' and 5'-GGT TTA GTG GTA CTT GTG AGC C-3'.

Whole body section of mouse embryos

Pregnant female and normal male ICR mice were kept under standard housing, feeding, and lightening condition (23℃, 12 h light/12 h dark cycle). On the appropriate day of pregnancy (9.5, 11.5, 13.5, and 16.5 day), mice were sacrificed by cervical dislocation and then embryos were dissected out. Embryos were fixed with 4% paraformaldehyde at 4℃ over-

night. After embedding into paraplast, sections (6-8 μm) were prepared and then mounted on polylysine-coated slides.

Generation of RNA probes for in situ hybridization

To generate CP2 riboprobe by *in vitro* transcription, 290 bp RT-PCR product of CP2 was subcloned into pCR™II vector (Invitrogen). The sequences and orientation of insert were verified by subsequent sequencing of the isolated clones. Sense RNA probe was generated by transcription with T7 RNA polymerase after recombinant plasmids were linearized with BamHI. For anti-sense RNA probe, the plasmids were linearized with XhoI and transcribed with SP6 RNA polymerase. Riboprobes were labeled with digoxigenin-11-UTP at the concentration of 350 μM/l in the transcription reaction using a protocol based on the manufacturer's instructions (Genius system, Boeringer Mannheim). The assessment of probe size and label incorporation was followed by native gel electrophoresis (Park et al., 1996) and ethidium bromide staining, or by relative anti-digoxigenin antibody staining to the control after blotting of serial diluted samples into the nitrocellulose membrane (Boeringer Mannheim).

In situ hybridization

In situ hybridization was performed as described (Martinez-Montero et al., 1991). Briefly, sectioned tissue slides were dewaxed by immersing into xylene and then gradually rehydrated in a series of ethanol, saline, and phosphate buffered saline (PBS) washes. After post-fixation with 4% paraformaldehyde, nucleic acids were unmasked by exposing tissue sections to proteinase K (50 μg/ml) for 10 minutes at 37℃. After proteolysis, sections were quickly rinsed in PBS, distilled water, and post-fixed in 4% paraformaldehyde, followed by quick rinses in PBS.

The sections were covered with 200 μl of prehybridization mix (50% formamide, 5×SSC, 0.1% Tween 20, and 100 μg/ml salmon sperm DNA) and then incubated in a moistened chamber for 2 h at 37℃. Slides were drained and 50 μl of hybridization mix containing digoxigenin labelled sense- or antisense probe (50 μg/slide), was pipetted on each slides. Tissue sections, after covered with siliconized glass coverslip, were incubated in 47℃ moistened chamber for 16 h.

After hybridization, slides were washed at high stringency in 0.1×SSC at 42℃ and then hybridized probe was detected with the alkaline phosphatase-coupled anti-digoxigenin antibody according to the pack insert protocol of Boeringer Mannheim. To inhibit endogenous non-intestinal phosphatase activity, levamisole (2.4 mg/10 ml, Sigma) was also included in the color solution.

Immunofluoresence microscopy

Globin assessment at the protein level was accomplished by antibody staining as described (Zitnik et al., 1993). Cultured MEL cells in the presence or absence of HMBA for 3 days were collected by centrifuge and then washed twice with PBS. Cell pellet was resuspended in two volumes of fetal bovine serum and then 2 μl of sample smear was made on the cleaned slide. Slides were dried in air for an hour. After fixing by placing in 50% methanol/50% acetone for 20 min, slides were dried in air and then sequentially rinsed in PBS and distilled water. Ten microliter of 1:750 diluted rabbit anti-mouse β-globin antiserum (Cappel, Pennsylvania) was applied to the marked area of the slide and then incubated for 1 h at 37℃ in humidified chamber. After sequential washing in PBS and water, 10 μl of 1:20 diluted FITC-conjugated goat anti-rabbit IgG antiserum (ICN

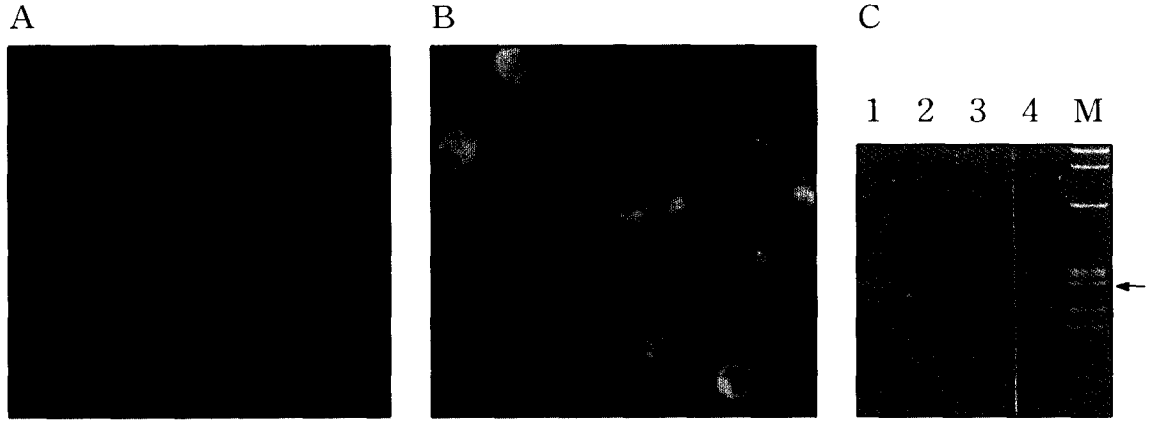

Fig. 1. CP2 messages increase 3 to 5 fold during the induced differentiation of MEL cells. Expression of β^{major} globin protein was analyzed in undifferentiated (A) and 3-day HMBA-induced (B) MEL cells by using FITC-conjugated antibody (400 x magnification). The amount of CP2 transcripts during HMBA-induced differentiation (C) was monitored by RT-PCR assay. M denotes *Msp*I digested pBR322 DNA size marker; lanes 1 through 4 denote HMBA-induction for 3, 2, 1, and 0 days, respectively.

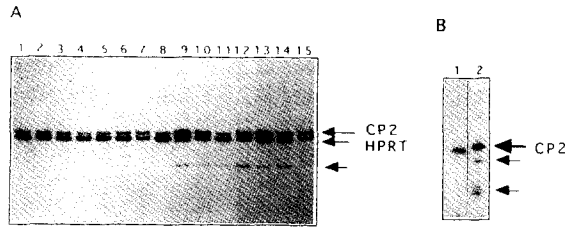

Fig. 2. CP2 messages are detected even in undifferentiated ES cells and the amount of CP2 messages is leveled up from day 8 of differentiation. A, total RNAs isolated from pools of about 50 EBs in the culture every 24 hours for 2 weeks, were used for RT-PCR analysis. After RT-PCR reaction for CP2 and HPRT, individually, by using each gene-specific primer set, equal amounts of each reaction were loaded together into the same well of 5% polyacrylamide gel for electrophoresis. Lanes 1 through 15 correspond to days of EB culture from 0 to 14. Arrows at the right hand represent the correct sized bands of CP2 and HPRT. Smaller sized bands from lanes 9 through 14 are marked by small arrow. B, to check the origin of the smaller sized band in panel A, each RT-PCR reactions for CP2 and HPRT was loaded in separate lanes in electrophoresis. Lane 1, HPRT; lane 2, CP2.

Biochemicals) was applied to each area and then incubated, washed, and dried as above.

Results

CP2 gene transcription increases during HMBA-induced terminal differentiation of MEL cells

Transcription factor CP2 was identified initially to bind the promoter region of the murine α-globin gene and its activity was shown to increase 2 to 3 fold during HMBA-induced differentiation of MEL cells. To monitor whether the increment of CP2 activity is caused by the up-regulation of transcription initiation in MEL cells, the steady-state level of CP2 transcripts was measured by RT-PCR assay. Total cellular RNAs were extracted from MEL cells as well as from HMBA-induced differentiating cells every 24 hours for 3 days. As shown in Fig. 1 (C), the amount of CP2 messages increased 3 to 5 fold during induced differentiation of MEL cells when equal amount of cDNAs were used for the PCR analyses. To make sure that globin synthesis was induced normally during differentiation of MEL cells in this study, aliquots of each cells were also characterized of their hemoglobin synthesis by immuno-staining with anti-murine β-globin antibody or by visual inspection of cell pellet (pink to red color appears in induced MEL cells). At the third day of induction, comparing to the uninduced cells, more than 95% of cells showed hemoglobinization (Fig. 1 A and B). Thus, it is most likely that the increment of CP2 activity during induced differentiation of MEL cells primarily depends on the increase of transcription initiation.

CP2 gene is expressed even in undifferentiated ES cells and the level of expression is modulated during differentiation

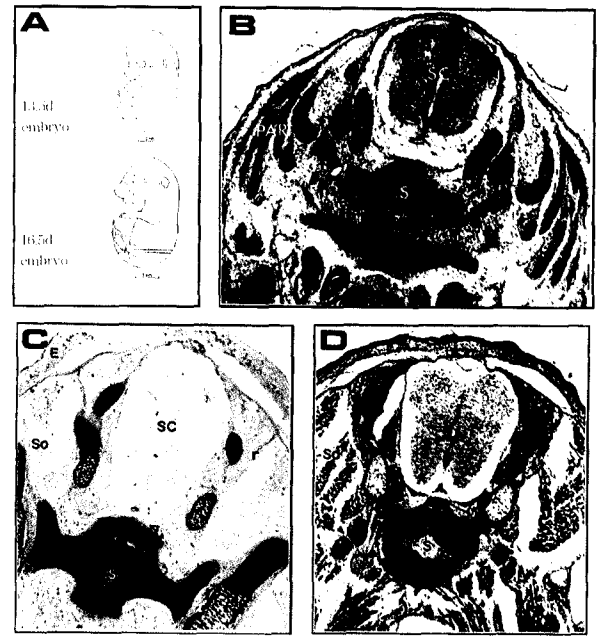

Fig. 3. Colorimetric detection of transcription factor CP2 mRNA on paraffin sectioned mouse embryos with digoxigenin-labeled CP2 ribo-probe. A, schematic drawing of 13.5 and 16.5 day mouse embryos after fertilization showing both the region and the direction of sections in the following panels. B, 13.5-day-old embryo section probed with antisense CP2 riboprobe. C, 16.5-day-old embryo section probed with CP2 sense probe. D, 16.5-day-old embryo section probed with CP2 antisense probe. PAR (PMC), premuscle mesodermal condensation; S, spine; SC, spinal cord; E, epithelium; So, somite. 50× magnification.

To gain more insight into the expression profile or modulation of expression of CP2 gene in early embryogenesis, *in vitro* differentiation system of ES cells was exploited. In previous studies, we set up a simple ES-based *in vitro* differentiation system that can dissect the molecular events of early embryogenesis (Kim, 1996a, b). In this improved system, not only all hematopoietic lineage cells are propagated efficiently and reproductively within the differentiating ES cells, but also the β-type globin switching in differentiating ES cells parallels the sequence of events found in the mouse embryo.

ES cells was differentiated to EBs to form and then total cellular RNAs were prepared from the pools of EBs every 24 hours for up to 2 weeks of the differentiation process (see Materials and Methods). The CP2 gene expression profile during this process was monitored by RT-PCR analysis, along with β-type globin and HPRT genes as internal controls. As shown in Fig. 2, CP2 transcript was observed even in undifferentiated ES cells (corresponding to the blastular stage in normal embryogenesis) and the level of expression increased about 3- to 5-fold from day 8 of the differentiation; indeed, normal mouse blastula also showed CP2 gene expression in RT-PCR analysis (data not shown). On the other hand, expression of β-type globin genes ap-

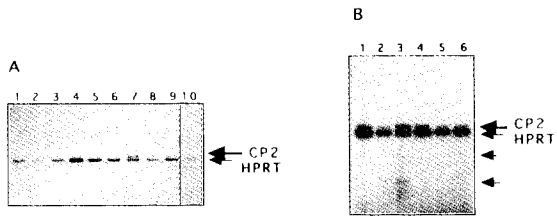

Fig. 4. CP2 expresses ubiquitously, but in different levels, in every mouse tissue tested. RT-PCR reaction was used to determine the presence or absence of CP2 mRNA in various tissues. A, CP2-specific band is rarely seen in the PCR reactions of 28 cycles. Lanes 1 through 10 represent heart, skeletal muscle, lung, forebrain, mid- and hind-brain, kidney, testis, ovary, placenta, and liver, respectively. B, CP2 could be detected readily in the PCR reactions of 35 cycles. Lanes 1 through 6 represent forebrain, mid- and hind-brain, testis, ovary, placenta, and heart, respectively.

peared to start from day 5 of differentiation (data not shown; Kim, 1996a, b). Thus, the data indicates that CP2 is rather an ubiquitously expressed transcription factor and even the major increase of CP2 transcript is not correlated with the onset of globin gene expression, which also suggests that there should be other tissues (or genes) requiring CP2 expression during normal development. It should be noted that the small sized RT-PCR products originated from CP2 specific primers also appear from day 8 through day 13 (Fig. 2B and see below).

CP2 gene is expressed in almost all of the embryonic tissues during early development

To monitor spatiotemporal distribution of CP2 messages during early embryogenesis, RNA *in situ* hybridization analyses to the mouse embryo tissues, at 11.5, 13.5, and 16.5 days after fertilization, were performed by using digoxigenin-labeled sense and antisense CP2 probes, respectively (see Materials and Methods). The indexing of developmental processes and emerging tissues followed (Kaufman, 1994). Even though there were differences in the level of expression among tissues, almost all of tissues showed positive signals to the antisense CP2 probe, but not to the sense CP2 probe, in all of the samples at different developmental stages. Representative sections, from 13.5 and 16.5 day mouse embryo, are illustrated in Fig. 3. As shown in the figure, positive signals to the antisense probe were observed in all tissues, while no positive signal was observed in the embryos treated with sense probe (Fig. 3C); endogenous alkaline phosphatase activity was exhibited strongly in the developing cartilage primodium even though alkaline phosphatase inhibitor (levamisole) was treated during the color development (see Materials and Methods). In 13.5-day embryo, positive signals to the antisense probe were prominent in the premuscle mesodermal condensations (PAR), ganglions and in the mantle and ependymal layers in spinal cord (Fig. 3B). On the other hand, in 16.5-day embryo, the prominent places were in ganglions, in

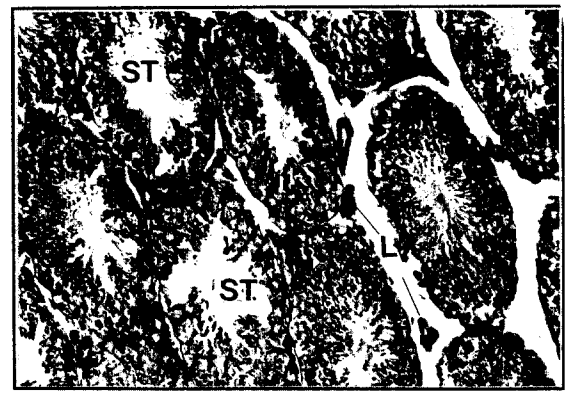

Fig. 5. Colorimetric detection of CP2 messages on the paraffin sectioned 3-month-old mouse testis with digoxigenin-labeled CP2 antisense probe. L, Leydig cells; SE, seminiferous epithelium; ST, seminiferous tubules. 200× magnification

multifidus and longissimus muscles which blend with the carcococcygeus dorsalis medialis muscle, and in psoas major muscle (Fig. 3D).

CP2 is also expressed in every mouse adult tissues examined and the strongest expression is in testis

Total RNAs extracted from several tissues of adult mouse were subjected to RT-PCR assay to determine the expression levels of CP2 transcript (Fig. 4). The CP2 mRNAs were expressed highly in rather restricted tissues of the adult animals even though it showed ubiquitous expression manner. In contrast to relatively abundant expression of CP2 in testis, ovary, and forebrain, a low level of CP2 expression was generally detected in every other tissues examined; the strongest expression appeared in testis and the level of expression was about as high as those of early differentiating ES cells *in vitro* (see Fig. 2).

To gain further insight into the possible involvement of CP2 in spermatogenesis, the spatiotemporal distribution of CP2 mRNAs was also investigated by RNA *in situ* hybridization to the tissue sections of 3-month old mouse testis (Fig. 5). Interestingly, strong positive signals were observed in Sertoli cells and interstitial cells including Leydig cells, but weak or no signals in the cells in spermatogenic process.

Discussion

To get more insight into the developmental and differentiation-dependent expression profile of CP2, steady-state levels of CP2 message were monitored in cultured MEL cells and differentiating ES cells *in vitro*, and in fetal and adult mouse tissues by using RT-PCR and *in situ* hybridization assays. At first, we showed that the increment of CP2 activity during induced differentiation of MEL cells is at the level of transcription (Fig. 1). Second, as implicated in previous studies on cell lines and mouse tissues by us

and others (Barnhart et al., 1988; Kim et al., 1990; Swendeman et al., 1994), CP2 expression is not restricted to the erythroid lineage cells, but rather expressed ubiquitously from the undifferentiated ES cells to adult tissue cells (Figs. 2 through 5). Finally, the level of CP2 transcripts is not uniform in tissues but rather modulates during development and differentiation, suggesting that high expression in specific tissue at specific developmental stage might be important for normal development.

Among several transcription factors that bind to the α-globin promoter, only CP2 shows increment of its transcriptional activity, at the transcription initiation level as shown in this study, during *in vitro* differentiation of MEL cells (Barnhart et al., 1988). Supplement of purified CP2 into the nuclear extract in the *in vitro* transcription analyses or overexpression of CP2 in MEL cells, activates the α-globin promoter templates in a factor binding site dependent manner (Kim et al., 1990; Lim et al., 1993), suggesting that CP2 is the factor directly involved in the transcription activation of α-globin gene. On the other hand, based on the following investigations, CP2 may also be involved in the transcriptional regulation of other globin genes as well as in general erythropoiesis. At first, CP2 can bind to the ε- and γ-globin promoters as a SSP heterodimer with a 45 kD partner protein (Jane et al., 1995), while CP2 has been shown to bind to the α-globin promoter as a homodimer (Lim et al., 1992, 1993; Yoon et al., 1994). Second, when MEL cells were stably transfected with a human antisense CP2 expression construct to reduce the steady state level of CP2 up to about an half of normal MEL cells, the transfected MEL cells showed lower basal expression of both α- and β-globin genes in uninduced state during HMBA induced differentiation (Kim C. G., preliminary data). On the other hand, the cells during the differentiation showed delayed increment of both α- and β-globin gene transcription (about 48 h). Surprisingly enough, the transcriptional delay was much more prominent in β-globin than in α-globin even though the CP2 binding sites were not identified in β-globin promoter. Thus, CP2, alone or along with other factors, seems to play important regulatory roles in globin gene expression and/or switching during erythropoiesis even though detailed mechanism is not at hand.

Besides globin switching, CP2 may be important in early development and differentiation of specific tissues. CP2 transcript was observed even in normal mouse blastula and undifferentiated ES cells, and the prominent up-regulation occurred in the 8th day of ES cell differentiation *in vitro* (Fig. 2), corresponding to about 12.5 to 13.5 day after fertilization in normal mouse embryogenesis (Wong et al., 1983; Kim, 1996a). It needs further characterization in normal early embryos to identify specific tissues that show prominent up-regulation of CP2. In the meanwhile, small sized bands, originating from the RT-PCR products of CP2, appeared in days 8 through 13 EBs, where CP2 was expressed in a much elevated level; similar situation was shown in the adult testis (Fig. 4). These extra PCR products can be interpreted as an experimental artifact, or as having derived from alternative splicing of CP2 gene or from closely related genes, but it should be characterized further. Moreover, along with this phenomenon, the interpretation of ubiquitous nature of CP2 expression should also be dealt with caution because, while only one kind of CP2 cDNA has been identified yet in MEL cells (Lim et al., 1992; Jane et al., 1995), 4 similar but distinct CP2 (LBP-1) cDNAs, by two related genes as well as alternative splicing, were identified and characterized in HeLa cells (Yoon et al., 1994).

CP2 is known to be homologous to the *Drosophila* transcription factor Elf-1/NTF-1 (Bray et al., 1989; Dynlacht et al., 1989). Elf-1/NTF-1 has been identified to a previously known embryonic lethal locus (grainyhead, grh; Bray and Kafatos, 1991), and implicated in the tissue-specific expression of several developmentally regulated *Drosophila* genes including *fushi tarazu* and dopa-decarboxylase gene (Dynlacht et al., 1989; Attardi and Tjian, 1993). Because Elf-1/NTF-1 is an essential transcriptional activator that functions during *Drosophila* embryogenesis, it is not surprising to speculate that CP2 also functions an essential role in early embryogenesis, and that the alteration of CP2 expression could have unanticipated consequences in specific tissues. Indeed, ES cells expressing antisense CP2 RNA controlled by CMV enhancer/promoter, did neither differentiate properly *in vitro,* nor form tumor when cells (1×10^6) were injected by the subcutaneous route into the 4-week- old nude mice (preliminary data, Kim et al.). Thus, the functional significance of CP2 during development should be reexamined; it should be included whether not only alternative spliced messages or closely related gene products are in charge, but also homo- or heterodimerization of CP2 with other tissue-specific or developmental stage-specific partners is involved. Obviously, the detailed expression profile of CP2 in early mouse embryogenesis, including the stages that show prominent structural changes, such as gastrulation, somatogenesis, and neurulation should also be included.

In summary, the increment of CP2 activity during induced differentiation of MEL cells is at the level of transcription, and the concentration balance between CP2 and/or the heterodimeric partner of CP2, along with other factors, might be important in globin switching during erythropoiesis. On the other hand, CP2 expression is not restricted to erythroid lineage cells as expected; CP2 is expressed ubiquitously from undifferentiated ES cells to adult tissue cells. However, the fact that level the of CP2 expression is variable among tissues during differentiation, suggests that

further intensive characterization should be done to validate the detailed functional role of CP2 in normal development.

Acknowledgements

We thank to Sang Dai Park, Eun-Ho Park, and Jong-Bok Yoon for their encouragements and valuable advices. This work was supported by KOSEF grant (951-0502010-2).

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[Received December 5, 1996; accepted February 20, 1997]