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Expression of SET Is Modulated as a Function of Cell Proliferation

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Abstract We explored a biological role of SET as it relates to cell proliferation and differentiation. Immunohistochemical staining demonstrated that the expression of SET was ubiquitous and diffuse over the whole embryo on gestational day 15. At a later stage of development, SET was expressed at relatively lower levels and localized to specific tissues and cells. On embryonic day 19, specific SET immunoreactivity was found in the epithelium of skin, respiratory tract, intestine, and retina as well as in muscle and cartilage. In these cells SET was stained mostly in the nucleus, which was supported indirectly by nuclear transport of enhanced green fluorescence protein-SET fusion proteins in ECV304 endothelial cells. *Set* mRNA expression was further confirmed in various cultured cells, including NIH 3T3 cells, L6 myoblast cells, human umbilical vein endothelial cells, and ECV304 cells. Using F9 teratocarcinoma cell lines, which were stimulated to differentiate into the two different cell lineages of parietal and visceral endoderm, we have further examined the role of SET. The expression of *set* mRNA and SET protein was diminished about three-fold in both differentiated endoderm cells compared to the undifferentiated F9 cells. However, when F9 cells were subjected to serum starvation, reduction of *set* mRNA abundance also took place at a similar level to that observed in response to differentiation. Consistent with this, quiescent L6 myoblast showed a marked downregulation of *set* mRNA compared to proliferating cells. These results suggest that SET is involved mainly in the regulation of cell proliferation rather than differentiation during embryonic development. J. Cell. Biochem. 74:119–126, 1999.

Key words: SET; embryo; F9 teratocarcinoma cell; proliferation

Set gene encoding a nuclear phosphoprotein [Adachi et al., 1994] is localized on chromosome 9q34 [von Lindern et al., 1992]. *Set* was found first in a patient of acute undifferentiated leukemia as a fusion of *set-can* by a somatic translocation [von Lindern et al., 1992]. Independently *set* was cloned from the cDNA library of the neonatal rat kidney and found to have two isoforms, α and β [Kim et al., 1994]. The sequence comparison between human [von Lindern et al., 1992], rat [Kim et al., 1994], and mouse (our unpublished data) revealed that *set* is highly homologous between these species, ≈94% same even at the nucleotide level, suggesting that SET may be important for certain cellular activities.

DNA replication of the adenovirus genome requires a host factor, template activating factor I (TAF-I), whose cloning revealed that it was identical with *set* [Nagata et al., 1995]. In addition, the nucleosome assembly protein NAP-1 [Ishimi et al., 1991] that was found to have higher homology in the acidic stretch of SET substituted for the TAF-I activity, indicating

Abbreviations used: EGFP, enhanced green fluorescence protein; HUVEC, human umbilical vein endothelial cell; NAP-1, nucleosome assembly protein-I; PP2A, protein phosphatase 2A; TAF-I; template activating factor-I.

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that the acidic region of these proteins is responsible for the promotion of viral replication *in vitro*. However, in supercoiling assay Fujii-Nakata et al. [1992] reported that the acidic stretch at the C-terminus of NAP-1 is not necessary for the intrinsic nucleosome assembly. Moreover, it is of interest to note recent observation that SET and NAP-1 specifically interact with mitotic B-type cyclins [Kellogg et al., 1995] and SET is a specific and potent inhibitor of protein phosphatase 2A (PP2A) [Li et al., 1996]. Since PP2A is known to be involved in a variety of cellular processes, potential role of SET could be expanded. However, it remains to be determined whether SET could act as an inhibitor of PP2A in vivo.

In mouse embryo *set* mRNA expression rapidly decreased during development [von Lindern et al., 1992]. The expression of *set* also seemed to be highly regulated during renal development [Kim et al., 1994] and tumorigenesis [Carlson et al., 1998]. Therefore, it was postulated that SET might be a regulatory factor in nephron morphogenesis. Taken together, these findings suggested a link between the regulation of cell proliferation or differentiation during development and a potential role of SET.

Since a model of renal morphogenesis was initiated by Grobstein [1953], this system has been modified and utilized extensively for the molecular analysis of development. However, it does not seem to be suitable to determine in this system whether the expression of SET is associated with an alteration in the state of cellular proliferation or differentiation. In the present study to further elucidate a role of SET, a defined model of F9 embryonal teratocarcinoma cells was chosen. We report here that the expression of *set* mRNA was reduced as a function of cell proliferation in F9 cells.

MATERIALS AND METHODS

Cell Culture

Mouse F9 teratocarcinoma cells were cultured as described [Hogan et al., 1981]. Differentiation into parietal endoderm was induced by addition of 10^{-7} M of retinoic acid and 10^{-3} M of dibutyryl cAMP. On the other hand F9 cells were differentiated into visceral endoderm by growing them in suspension in the culture medium containing 10^{-7} M of retinoic acid. For serum starvation F9 cells were maintained in the medium containing 0.2% fetal bovine serum (FBS) for 3 days [Finklestein et al., 1988]. Rat L6 myoblast and NIH 3T3 cells from Korean Cell Line Bank and ECV304 cells from American Type Culture Collection (Rockville, MD) were obtained and maintained as recommended. Human umbilical vein endothelial cells (HUVECs) were provided by Dr. Kim S.Y. (Chungbuk National University, Cheongju, Korea). L6 myoblasts were serum starved in 0.5% FBS for 24 h.

Production of GST-SETβ Fusion Proteins

The open reading frame (ORF) of *set* cDNAβ was amplified with PCR using the cloned cDNA [Kim et al., 1994] as template, followed by subcloning into BamH I and EcoR I site of pGEX-2T plasmid (Pharmacia, Uppsala, Sweden). The PCR conditions were for 30 cycles in denaturation, 94°C, 1 min; annealing, 55°C, 1 min; extension, 72°C, 1 min. The primer sequences were as follows:

5', -CCCGGATCCATGTCTGCGCCGACGGCCAAA;3', -CCCGAATTCGTCATCCTCGCCTTCATCCTC.

Esherichia coli (DH5α) transformed with recombinant pGEX-2T plasmids were induced to overexpress GST-SETβ fusion proteins by incubation for 5 to 7 h in the presence of 0.1 mM isopropyl-β-D thiogalactoside (IPTG). The fusion proteins were purified as described previously [Donald et al., 1988]. Purified recombinant products were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

Generation of Antibodies

Polyclonal antibodies were raised against the purified SETβ protein in rabbits as described [Copeland et al., 1986]. These antibodies could detect both SETα and SETβ, since most of sequences for α and β are same, except for a short N-terminal fragment [Kim et al., 1994].

Immunoblotting

Immunoblot analysis was carried out according to the published procedures [Salinovich et al., 1986]. The blot was incubated with a 1:200 dilution of polyclonal anti-SETβ IgG, then with 1:10,000 dilution of a peroxidase-conjugated donkey anti-rabbit IgG (Amersham, Buckinghamshire, UK). The signal was detected using an ECL kit (Amersham).

Northern Blot Analysis

Total RNA was isolated from frozen embryos and cultured cells by the guanidine thiocyanate

method [Chomczynski et al., 1987]. Hybridization was performed under high-stringency conditions as described [Kim et al., 1994]. Thirty or 40 μg of total RNA per lane was loaded. To confirm the proper differentiation of F9 cells the blot was probed with laminin cDNA. For equal loading of RNA aldolase or GAPDH probe was used. The autoradiographs were analyzed by a densitometer (Molecular Dynamics, Sunnyvale, CA). The same experiment was carried out more than two times and the representative data were presented here.

Immunohistochemistry

Immunohistochemistry was done using an immunoperoxidase technique as described previously [Paxinos et al., 1980]. Briefly, paraffin sections were deparaffinized through xylene and graded alcohol series. Endogenous peroxidase activity was quenched by 0.3% H_2O_2. The sections were incubated with anti-SETβ (1:200 dilution), followed by incubation with biotinylated anti-rabbit antibody (1:200 dilution) and Vectastatin ABC reagent (Vector Laboratories, Burlingame, CA). Finally, the peroxidase reaction was visualized by incubating with 0.05% DAB in 0.02 M phosphate buffered saline (PBS) containing 0.003% H_2O_2. When molar excess (×100) of SETβ was added, no significant staining was seen.

Plasmid Vector and Transfection

pEGFP-SET expressing enhanced green fluorescent protein (EGFP)-SET fusion proteins was constructed as follows. cDNA encoding SETβ was subcloned into Hind III–EcoR I sites of pEGFP-C2 (Clontech, Palo Alto, CA). Then to make SETβ in-frame to EGFP this vector was digested with Xho I that is located between GFP and set cDNA, blunt ended with Klenow enzyme, followed by ligation. In-frame fusion and correct sequence of *set*β was verified using a T7 Sequenase DNA sequencing kit (Amersham). ECV304 endothelial cells were seeded onto the sterilized cover slips in 60 mm tissue culture plates (1×10^6/well) 1 day prior to transfection, and grown in M199 containing 10% FBS. At 70% confluency cells were transfected with 5 μg of pEGFP-SET or pEGFP-C2 vector DNA (control), respectively, using the calcium phosphate reagent (Invitrogen, San Diego, CA). At the end of transfection the coverslip was removed from the plate and washed three times with PBS. Then, freshly made PBS/4% paraformaldehyde (1 ml/coverslip) was added directly on the coverslip, followed by incubation for 15 min at 4°C. After being washed twice with PBS, the coverslip was mounted onto a glass slide. Fluorescence was visualized under a laser confocal microscope (Model No; MRC-1024, Bio-Rad Laboratories, Richmond, CA).

RESULTS

Expression of SET During Mouse Embryonic Development.

To perform the immunohistochemical analysis SETβ was overexpressed, purified, followed by generation of antibodies. Purified SETβ migrated at 37 kDa slower than the predicted size of 32 kDa calculated from the composition of amino acids (Fig. 1D). Polyclonal antibodies were generated in rabbits and were verified with a Western blot (data not shown).

To determine whether the localization and the expression level of SET change during embryogenesis, immunohistochemistry, and Western blot were applied. SET was expressed highly in the embryos and gradually decreased during development (Fig. 1A). At the earlier stage, SET has been found in most organs and tissues examined, although the relative level of expression varied. As development proceeds, SET seemed to be localized. On embryonic day 19, immunoreactive SET was present in epithelial cells, fibroblasts, chondrocytes, smooth muscle cells, and endothelial cells. SET was found to be predominantly nuclear in these cells. Immunoreactivity of SET was intense generally in the epithelial cells of skin, nasal cavity, intestine, and developing retina (Fig. 1B) as well as in chondrocytes (data not shown). Interestingly, SET was specifically stained in the basal layer of epidermis, in which proliferation is taking place actively, but rare in other layers. In the adult skin a similar pattern was observed. However, many cells of stratum spinosum were also strongly stained (data not shown). Among the adult organs, testis was one of the highest to express SET, while in liver no significant staining was seen (Fig. 1C). In testes, most of spermatocytes and spermatids showed strong reactivity, while in a part of spermatogonia SET staining was spared. Western blot was consistent with the immunohistochemical data, that the expression of SET was four-fold greater in the embryo of day 15 compared to that of day 19 (Fig. 1D). The weaker bands above SETβ, pre-

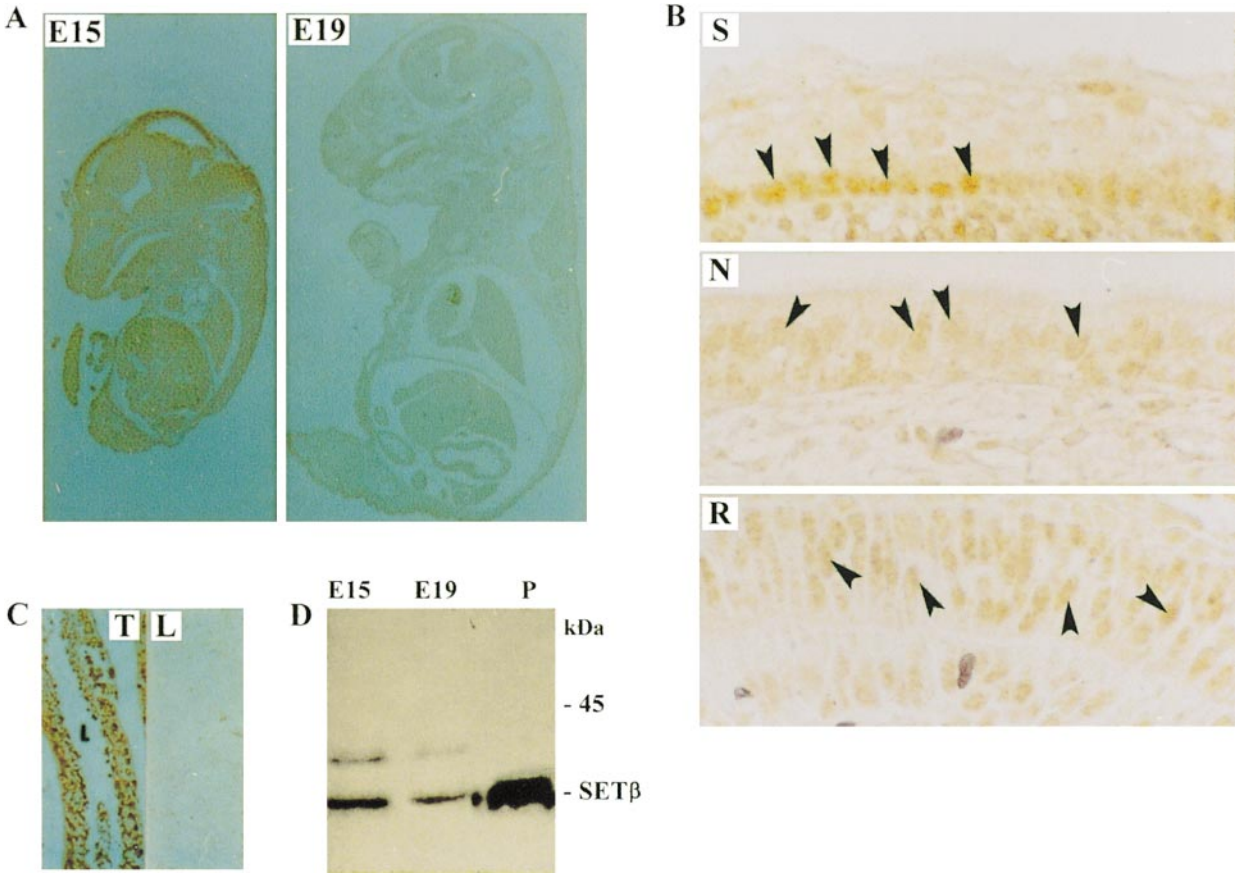

Fig. 1. Expression and localization of SET in mouse embryo. **A**: Immunohistochemistry of mouse embryo with anti-SET IgG at day 15 (left) and day 19 (right) of gestation, respectively. **B**: Composite picture of epithelium from skin (S), nasal cavity (N), and developing retina (R) stained for SET. Some cells expressing SET are indicated (arrow head). Magnification, ×400. **C**: Immunohistochemical analysis of adult testis (left) and liver (right). L, luminal side. Magnification, ×400. **D**: Lysate (100 μg) from mouse embryo was subjected to immunoblot analysis with anti-SET IgG. E15, embryo at day 15; E19, embryo at day 19; P, purified SETβ.

sumably derived from α isoform of *set*, were also seen in both embryos of day 15 and 19.

Nuclear Localization of SET in ECV304 Endothelial Cells

To confirm the nuclear transport of SET transient transfection analysis was performed with ECV304 endothelial cells. Localization of SET was monitored indirectly by fluorescence of the EGFP-SET fusion proteins. When transfected with the control vector, nonspecific fluorescence of irregular shape was observed (Fig. 2a). By contrast, transfection of the EGFP-SET expression vector produced strong specific signals in the cells. EGFP-SET fusion proteins were readily detectable early at 4 h after transfection (data not shown). At this stage SET was evenly distributed in the cytoplasm. After 8 h SET was present both in the cytoplasm and the nucleus (Fig. 2b). However, the relative intensity was much stronger in the nucleus. At 24 h most of EGFP-SET proteins were positive in the nucleus

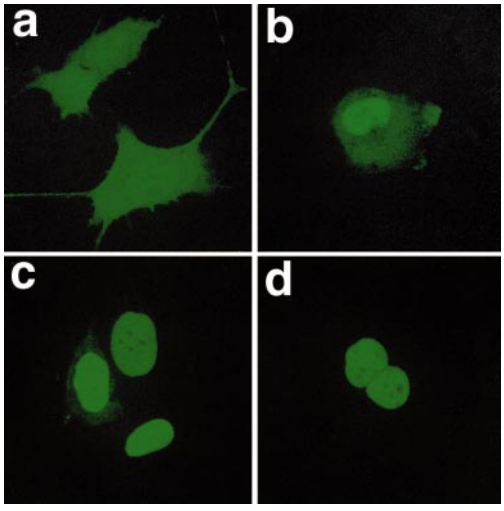

Fig. 2. Visualization of EGFP-SET proteins in ECV304 endothelial cells. ECV304 cells were transfected with the control vector (pEGFP-C2) or the EGFP-SET expression vector (pEGFP-SET) and analyzed by confocal microscopy. Control at 24 h after transfection (**a**), EGFP-SET expression at 8 h (**b**), 24 h (**c**), and 48 h (**d**).

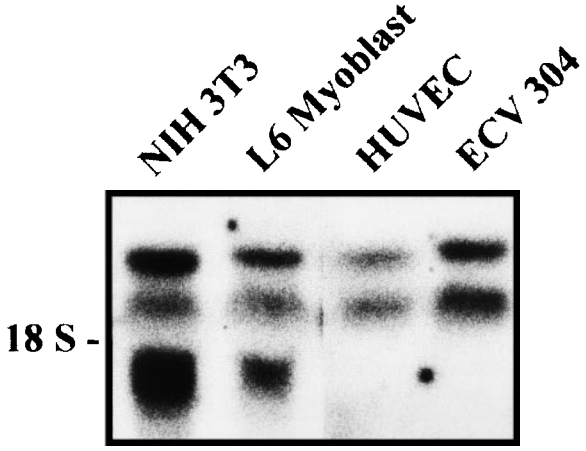

Fig. 3. Expression of *set* mRNA in cultured cells. Total RNA was isolated from cultured NIH 3T3 cells, L6 myoblast, HUVEC, and ECV304 cells. Forty micrograms of total RNA was separated on 1.2% agarose gel, and transferred to a nylon membrane. Hybridization was performed with [^{32}P-dCTP] labeled *set* cDNA. RNA size markers are shown on the left.

with faint staining in the cytoplasm of some cells (Fig. 2c). As shown in Figure 2d, EGFP-SET proteins remained persistently in the nucleus until 48 h.

Expression of *set* mRNA in Cultured Cells

To validate the immunohistochemical findings in vivo, *set* mRNA expression was examined in cultured cells. As representative cells for myoblast, fibroblast, and endothelial cells L6 myoblast, NIH 3T3 cells, HUVEC, and ECV304 cells were chosen, respectively. As shown in Figure 3, *set* mRNA was expressed in all four cells examined. In human cells of HUVEC and ECV304 two major transcripts of 3.0 kb and 2.2 kb were identified, whereas in rat L6 myoblast and mouse NIH 3T3 cells three transcripts of 3.0 kb, 2.2.kb, and 1.6 kb were detected. In ECV304, an endothelial cell line derived from HUVEC by spontaneous transformation, set mRNA was upregulated compared to HUVEC.

Effect of Cellular State on *set* mRNA and Protein Expression

To determine whether the expression of *set* is regulated by the altered state of cellular differentiation, F9 cells were induced to differentiate into parietal and visceral endoderm cells. Then total RNA and proteins were isolated and subjected to northern and western blot analysis, respectively. Differentiation of F9 cells was evaluated by the characteristic morphology [Hogan et al., 1981] and the induction of another differentiation marker, laminin [Strickland et al., 1980] (Fig. 4A, center). In a Northern blot *set* mRNA was consistently shown as two bands, 3.0 kb and 1.6 kb in size. These two transcripts were expressed in a similar pattern. In undifferentiated F9 cells *set* mRNA was highly expressed, but gradually downregulated to about one-third in parietal and visceral endoderm cells (Fig. 4A, top). This was in agreement with the result of a Western blot, showing that expression of SET decreased, to a similar extent of three-fold, with differentiation into parietal and visceral endoderm (Fig. 4B).

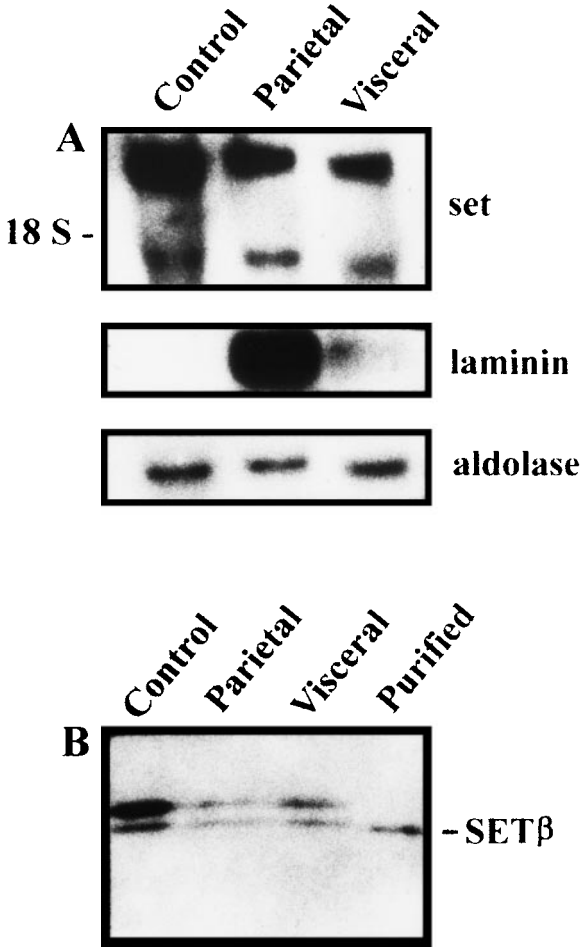

Fig. 4. Altered expression of *set* mRNA and protein in differentiated F9 cells. F9 cells were induced to differentiate into parietal and visceral endoderm cells as described in Materials and Methods. **A**: Total RNA was isolated from untreated (control) and treated (parietal and visceral) F9 cells. Thirty micrograms of total RNA per lane was loaded. The blot was hybridized with a [^{32}P-dCTP] labeled *set* cDNA. The same blot was reprobed with laminin (center) and aldolase cDNA (bottom). **B**: Immunoblot analysis was performed with the lysate (75 μg) from control, parietal and visceral F9 cells with anti-SET IgG.

To examine the possibility that this may occur as the result of cellular quiescence associ-

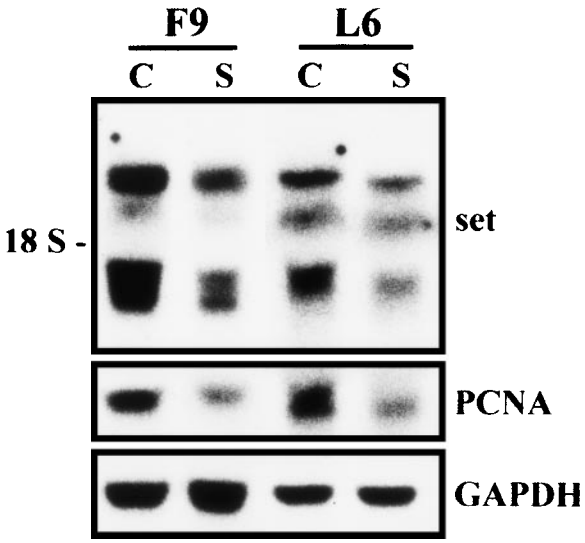

Fig. 5. Altered expression of *set* mRNA in proliferating and quiescent cells. Total RNA was isolated from control (C) and starved (S) F9 cells and L6 myoblasts. Forty micrograms of total RNA per lane was loaded. Hybridization was performed first with labeled PCNA probe as described above. The same blot was reprobed with *set* cDNA (top) and GAPDH for equal loading (bottom).

ated with differentiation, F9 cells were serum starved. PCNA, an index of cell proliferation, was downregulated as expected, showing that the cells were correctly manipulated (Fig. 5, center). Under this condition reduction of *set* mRNA was greater, four-fold, than induced by differentiation. Furthermore, when rat L6 myoblast was subjected to the same analysis, *set* mRNA was also markedly reduced (Fig. 5 top).

DISCUSSION

This study describes the expression of the gene encoding SET during mouse development and in F9 embryonal teratocarcinoma cells in response to differentiation or serum starvation. The findings presented here indicate that SET may be a regulator for cell proliferation rather than differentiation.

Although it is well known that SET is involved in leukemogenesis through formation of a fusion gene with CAN, its normal cellular function has not been clearly demonstrated yet. *set* mRNA was found to be highly expressed during the early phase of mouse development [von Lindern et al., 1992] and kidney morphogenesis in rat [Kim et al., 1994], suggesting that it may have specific functions during multicellular development. Our immunohistochemical data and Western blot in mouse embryos are consistent with this idea. SET expression was high in the earlier stage of embryo, gradually decreasing during development. Although SET expression was ubiquitous in the early phase, later it was more restricted. It is of interest to note that epithelial cells, in general, showed specific reactivity to anti-SET. Given the diverse functions executed by these cells and the ubiquitous expression of SET, this raises a possibility that SET may perform a common cellular activity depending upon the cellular state. Otherwise, SET expression observed in the various cell types, including chondrocytes, fibroblasts, and muscle cells, could not be explained easily. This is in agreement with a recent report that SET has a potent and a specific inhibitory activity toward PP2A whose tissue distribution is ubiquitous [Li et al., 1996]. Thus, it seems that SET could play a general role, rather than cell-type specific activity during development when it is expressed high enough.

Regarding a potential role of SET, skin seems to be a good model to determine whether SET is involved in cell proliferation or differentiation. On the embryonic day 19 epidermis is composed of multilayers, where proliferation and differentiation of keratinocytes are separated spatially. Proliferation is active mainly in the cells of the basal layer, while differentiation is undergoing consecutively in the upper layers [Morris et al., 1987; Furukawa et al., 1992]. SET was expressed mostly in the basal layer, although some cells in the upper layers and fibroblasts in dermis showed positive staining. These indicated that a function of SET may be closely associated with cell proliferation. In the adult skin, although a similar gradient of SET expression noticed in embryo was evident, higher proportion of spinous cells also showed stronger immunoreactivity, suggesting that SET may have a distinct role in the adult skin. Alternatively, SET could be replaced by a member of SET-like proteins in the adult, given that SET may comprise a family of proteins [von Lindern et al., 1992; Kim et al., 1994; Vaesen et al., 1994]. The finding that localization of NAP-1, a member of SET family in yeast, is predominantly cytoplasmic [Kellogg et al., 1995] strongly argues that there are more unidentified members in this family. A proliferative function of SET could be examined in liver, in which SET expression is negligible. When liver is subjected to partial hepatectomy, active cell proliferation is taking place within 24 h. It would be

interesting to examine whether SET expression is upregulated under this condition.

To further explore a biological role of SET related with cell proliferation or differentiation, its expression was examined in cultured F9 embryonal teratocarcinoma cells under various culture conditions. Since F9 cells do not differentiate spontaneously [Hogan et al., 1981], only by the differentiating agents of cAMP and retinoic acid, they have been frequently used for analysis of differentiation or proliferation-associated cellular responses. *set* mRNA and protein were abundant in undifferentiated F9 cells, and decreased during differentiation into parietal and visceral endoderm, raising the possibility that SET is associated with differentiation of F9 cells. However, rendering them quiescent with serum starvation, *set* mRNA was reduced to a similar extent, suggesting that the decrease in SET expression during differentiation may be due to a halt in cell cycle rather than associated with differentiation *per se*.

In this context, earlier results suggesting diverse roles for SET related with nuclear events seem to be pertinent. Namely, higher homology of SET with the *S. cerevisiae* nucleosome assembly protein NAP-1 [von Lindern et al., 1992], and interaction of SET with B-type cyclins during mitosis [Kellogg et al., 1995]. In addition, SET-CAN fusion proteins were shown to be present always in the nucleus. It is not clear, however, whether SET played a major role in relocation of C-terminal part of CAN, since there is still a controversy on the localization of CAN itself, cytoplasmic [Kraemer et al., 1994] or nucleoplasmic [Fornerod et al., 1995] side of the nucleopore complex. Our immunohistochemistry in mouse embryos demonstrated that SET was mainly nuclear in the various tissues and cells. Furthermore, nuclear transport of SET was confirmed using an EGFP marker in endothelial cells. These results are consistent with the previous data, which showed nuclear localization of SET by indirect immunofluorescent staining [Adachi et al., 1994]. Given that in vitro SET is a potent inhibitor of PP2A [Li et al., 1996] which has a negative effect on the progression from G2 to M phase by dephosphorylation of cdc25 [Clarke et al., 1993] and inhibition of cdk1 phosphorylation at Thr-161 [Lee et al., 1994] and is localized in cytoplasm as well as in nucleus [McCright et al., 1996], SET could regulate cell cycle. It is plausible via formation of a multi-complex, which consists of cyclin B, cdk1, and SET, since SET is known to interact with cyclin B [Kellogg et al., 1995]. Thus, SET could promote the transition of cell cycle to M phase by temporary inhibition of PP2A. The effect of SET activity on cell cycle would be terminated automatically by destruction of cyclin B. It remains to be determined whether this scenario works in the mammalian system.

Although SET seems to be closely linked with cell proliferation, it is not completely excluded that SET is, in part, involved in differentiation or other cellular activities. The phenotype of the leukemic cells carrying the *set-can* fusion gene in a patient with acute undifferentiated leukemia was reported to be very immature [Adriaansen et al., 1991], suggesting that SET-CAN may somehow impair the differentiation pathway and maintain the undifferentiated state. Downregulation of SET in F9 cells during differentiation also supports this notion. Further evidence that SET has a potential role, unidentified so far, was provided in testis. As an adult organ testis is unique, since it is undergoing maturation even in adult as well as expressing SET in a higher level. In the seminiferous tubule, spermatogenic cells of spermatogonia, spermatocytes, and spermatids are successive stages in a continuous process of differentiation. As spermatogenesis proceeds, immature spermatogonia are transformed into spermatids. PCNA staining revealed that the strong immunoreactivity was mostly found in the nuclei of spermatogonia [Chapman et al., 1994; Kang et al., 1997]. Surprisingly, SET was expressed highly in most cells, sparing some of spermatogonia, indicating that SET behavior is not parallel with PCNA. However, further studies are necessary to determine a role of SET in testis.

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