Function and regulation of *Oct-3* gene in mammalian early embryogenesis

哺乳動物初期発生におけるOct-3遺伝子の機能と発現制御

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学生証番号　17314

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Contents

Introduction

Introduction

During embryonic development of vertebrates, pluripotent stem cells are allocated to separate lineages and each cell lineage pursues a limited and stereotyped cell fate. It is considered that many parts of this process are controlled by multiple families of transcription factors. In fact, several such factors in vertebrate have been identified and analyzed for their functions. However, most of the factors are expressed and function in the embryos after gastrulation, a period when cell fates become determined. The earliest cell-lineage specification that occurs at around gastrulation is less understood at the molecular level at present.

In an effort to isolate transcription factors active at pre-gastrulation stages in vertebrate development, the first candidate gene *Oct-3* has been identified (Okamoto et al., 1990; Schöler et al., 1990a; Rosner et al., 1990). The *Oct-3* gene is expressed exclusively in toti-pluripotent stem cells of the pregastrulatory mouse embryo and germ cells (Rosner et al., 1990; Schöler et al., 1990a). It is also expressed in embryonic stem (ES) and embryonal carcinoma (EC) cells, but is rapidly switched off when these cells are induced to differentiate(Okamoto et al., 1990; Schöler et al., 1990a; Rosner et al., 1990). The *Oct-3* gene belongs to a large family of POU domain transcription factors. These proteins possess the conserved sequence motif called the POU domain as a DNA binding domain, but the remaining portions are quite diverse (Herr et al., 1988). Several genes encoding POU transcription factors have been identified in vertebrate and in invertebrate. Some of these are implicated in cell-fate determination (reviewed by Rosenfeld, 1991; Verrijzer and Vliet, 1993). Other members of this family are also expressed in undetermined embryonic cells. The Tst-1/SCIP/Oct-6 gene product appears early in development in the inner cell mass of mouse embryos, but

subsequently is expressed in specific cell types such as testis and myelinating glia (He et al., 1989; Monuki et al., 1990; Suzuki et al., 1990). *Xenopus laevis Oct-60* (Hinkly et al., 1992; Whitfield et al., 1993) and zebrafish *pou2* (Takeda et al., 1994) are closely related to *Oct-3* . They are maternally expressed, and the transcripts are present from the one-cell stage to the gastrula stage. Furthermore, it has been suggested that zebrafish pou2 is involved in the proliferation of blastmeres in undetermined state at the blastula stage and/or the early cell commitment events at the gastrula stage (Takeda et al., 1994). Although there is no direct evidence that the *Oct-3* gene plays a role in early embryogenesis, it may be required for maintaining pluripotency and that the down-regulation of the *Oct-3* gene may be required for subsequent differentiation.

In this study, I tested the role of Oct-3 in EC cell differentiation by using somatic cell hybrid system between EC cells and fibroblast cells (Chapter I), and then, analyzed the molecular mechanisms of Oct-3 extinction in EC cell differentiation (ChapterII)

Chapter I

Hybrid cell extinction and re-expression of *Oct-3* function correlates with differentiation potential

Introduction

To characterize the early events in cell-fate determination, EC cell lines have been often used instead of mouse embryo, since EC cells resemble embryonic stem cells in many ways. I used the P19 EC cell line as a model system for early embryogenesis. *Oct-3* is expressed in P19 cells, but is rapidly switched off when the cells are induced to differentiate (Okamoto et al., 1990). A simple approach to test the role of *Oct-3* in EC cell differentiation would be to establish P19 cell lines that can constitutively express *Oct-3* , and to examine their phenotype. However, there were two major problems with this approach. First, using conventional selection methods, we have been unsuccessful in obtaining stable cell lines expressing an exogenous *Oct-3* gene. Second, the Oct-3 protein alone is unable to activate an octamer-enhancer, due to its cell-type-specific activation domain (as described in result), indicating that the Oct-3 protein needs another factor to exert its function.

In order to overcome these problems, I have devised a system that can select cell depending on the presence or the absence of Oct-3 transactivating function. First, P19 cells that had been transformed with enhancer-trap were fused to L cells (mouse fibroblast), and hybrid cell lines were established. The hybrid cells were found to have differentiated into neuroepthelial-like cells, and accordingly the *Oct-3* gene expression was extinguished. I then introduced the Oct-3 transactivation function into the hybrid cells, and examined the phenotypic changes induced by the Oct-3 function.

Results

Experimental strategy

The experimental strategy for selecting the hybrid cells and their derivative cell lines is summarized in Figure I-1. Generally, when two different cell types are fused, expression of many cell-type specific genes is repressed in the hybrid cells (Davidson, 1974; Weiss et al., 1975). This phenomenon is known as hybrid cell extinction. I therefore expected that when P19 cells (Oct-3$^+$) were fused to fibroblasts (Oct-3$^-$), the *Oct-3* gene might be extinguished, and the hybrid cells might show certain differentiated phenotype. From the hybrid cells, I wished to isolate 'revertants' that had regained *Oct-3* function. In order to facilitate the selection of such 'revertants', a subline of P19 cells (052) was used as a parental cell line. 052 cells contain a single copy of an enhancer-trap (Bhat et al, 1988). The neo gene in the enhancer-trap is activated in P19 cells by an endogenous enhancer E1, which is a cell-type specific enhancer that requires the enhancer-activating function of Oct-3. As a result, expression of the *neo* gene is strictly dependent on Oct-3. Therefore, in a series of hybrid cells and their derivatives, cells would become G418-resistant only when Oct-3 transactivating function exists; the G418- resistance is directly based on the transactivating capability of Oct-3. In order to facilitate the selection of genuine hybrid cells, HGPRT-deficient 052 cells and thymidine kinase (TK)-deficient L cells were chosen as parental cell lines for cell fusion.

The hybrid cells differentiated to cells resembling neuroepithelial stem cells

HGPRT-deficient 052 cells and TK-deficient L cells were fused, and hybrid cells were selected in HAT medium. All the HAT-resistant colonies showed the same morphology. Unlike the parental cell lines, the hybrid cells had a large cell body and multiple long processes (Figure I-2). The colonies were

recovered and established as 20 independent cell lines. These hybrid cell lines were morphologically indistinguishable from each other(representative phase contrast photographs of four hybrid cell lines are shown in Figure I-2). They were stably maintained over several passages without morphological changes. Southern blot hybridization analysis with the enhancer-trap plasmid as a probe indicated that the hybrid cells retained the 052-derived *neo* gene (Figure I-3). Their morphology prompted us to examine various markers known to be specific to neural cells. Two such markers, nestin and Brn-2, were expressed. Nestin is an intermediate filament protein specifically expressed in neuroepithelial stem cells (Frederiksen and Mckay, 1988; Lendhal et al, 1990). The antibody raised against nestin stained the intermediate filaments of the hybrid cells (Figure I-4).

Brn-2 is a class III POU transcription factor expressed in developing and adult brain (He et al., 1989; Hara et al., 1992). It is not expressed in undifferentiated P19 cells, but is strongly induced when the cells differentiate into neural cells (Fujii and Hamada, 1993). Gel shift analysis of nuclear extracts from hybrid cells detected Oct-1 and another binding factor (Figure I-5A, lane F1, F2 and F3). The latter octamer-binding factor is most likely to be Brn-2, because an antibody against Brn-2 could recognize and super-shift the binding factor (Figure I-5B).

I examined the hybrid cells for other markers including glial fibrillar acidic protein (GFAP) and neurofilament protein, but they were negative. Taken together, these results suggest that the hybrid cells resemble neuroepithelial stem cells.

Oct-3 expression is extinguished in the hybrid cells

I next examined the expression of the *Oct-3* gene in the hybrid cell lines. First, Oct-3 protein level was assayed by Western blotting with an antibody raised against Oct-3. This antibody recognized a 43 kDa protein (Oct-3) in

undifferentiated P19 cells but failed to detect it in retinoic acid (RA)-induced P19 cells (Figure I-6A). The 43 kDa protein disappeared 2 days after RA-induction, which was consistent with the kinetics of the loss of *Oct-3* mRNA (Okamoto et al., 1990). The 43 kDa protein was not detected in non-EC cells such as HeLa and L-TK$^-$ cells, but was detected when an Oct-3 expression vector was transfected into HeLa cells (Figure I-8). Therefore, the antibody appears to be specific to Oct-3 protein. When the level of Oct-3 protein in the hybrid cells was examined with the antibody, Oct-3 protein was undetectable in any of the 20 hybrid cell lines (Figure I-6B).

The level of Oct-3 protein was also determined by gel-shift assay, and it was again undetectable in the hybrid cell lines (Figure I-5A, lanes F1~F3). Finally, *Oct-3* mRNA level was examined by Northern blots. While *Oct-3* mRNA was abundant in P19 cells, it was undetectable in the hybrid cell lines as well as in L-TK$^-$ cells (Figure I-7). Due to the lack of Oct-3 expression, all the hybrid cell lines were sensitive to G418.

These results, taken together, indicate that the expression of *Oct-3* gene is extinguished in the hybrid cells at the transcriptional level.

Oct-3 modulating activity is also extinguished in differentiated cells

It has been established by others (Rosner et al., 1990; Schöler et al., 1990a) that Oct-3 acts as a transactivator in HeLa cells when the octamers are placed closed to the TATA box. In this study, I have examined whether Oct-3 can stimulate the enhancer-dependent transcription from a distal binding site. When the octamers (26bp sequences derived from E1) were placed 0.1 kb away from the SV40 promoter, these sequences act as a cell-type specific enhancer; pOCTAcat, relative to pBScat, was transcriptionally active in P19 cells (Figure I-8, lanes 1 and 2), but inactive in HeLa and F1 cells (Figure I-8, lanes 3, 4, 10 and 11). When the Oct-3 expression vector was co-transfected

into F1 or HeLa cells along with pOCTAcat, the Oct-3 expression vector was unable to stimulate octamer-dependent transcription (Figure I-8, lanes 5~7 and 12~14). pCMVOct-3 in fact repressed the basal level of transcription in these cells. This observation is analogous to the finding by others (Lenardo et al., 1989) that Oct-3 (then called NF-A3) negatively regulates the immnoglobulin enhancer in EC cells. Western blot analysis confirmed that a considerable amount of Oct-3 protein was produced in the transfected cells (Figure I-8, lanes 8 and 9). Furthermore, Oct-3 protein produced in HeLa cells was capable of binding to the octamer sequence (Schöler et al., 1991). Therefore, the failure of Oct-3 to stimulate enhancer-dependent transcription in the differentiated cells appeared to be due to the inactivity of its transactivation domain. To confirm this, the Oct-3 transactivating domain, the amino-terminal region of this protein (Okamoto et al., 1990; Imagawa et al., 1991) was fused to the DNA-binding domain of GAL4. Such a chimeric protein (GAL-4/Oct-3) was able to activate expression of an appropriate reporter plasmid (1xUAScat) in P19 cells, but failed to do so in L-TK$^-$ and F1 cells (Figure I-9). GAL-4/VP16, in which the GAL4 DNA-binding domain was fused to the HSV-VP16 transactivating domain, could transactivate the reporter in the hybrid cells as well as in L-TK$^-$ and P19 cells. These results now show that the transactivating domain of Oct-3 protein stimulates the enhancer-dependent transcription in a strictly cell-type-specific fashion. It appears that, upon cell fusion, not only expression of the *Oct-3* gene itself but also the activity required for function of the Oct-3 activating domain are extinguished.

The Oct-3 transactivating function induces dedifferentiation of the hybrid cells

The hybrid cells did not express the *Oct-3* gene (Figure I-5A, 6B and 7). Therefore, the endogenous *neo* gene was inactive and hybrid cell lines were sensitive to G418 (Table I-1). If some of the hybrid cells regain Oct-3

function, such cells ('revertants') should become resistant to G418. Initially, I simply transferred the F1 cells to G418-containing medium but no spontaneous 'revertants' were recovered; the frequency of spontaneous reversion was less than 1 in 2 x 10^7 cells. Subsequently, *Oct-3* was ectopically introduced into the hybrid cells. When the F1 cells were transfected with the Oct-3 expression vector (pCMVOct-3) alone and then cultured in the presence of G418, no G418-resistant cells were rescued (less than 1 in 2 x 10^7 transfected cells). This was not surprising, since ectopically expressed Oct-3 alone could not stimulate enhancer-dependent transcription in differentiated cells including the hybrid cells (Figure I-8 and 9). It should be noted that the E1 enhancer is located 1 kb upstream of the *neo* gene promoter. Schöler et al. (1991) have shown that Oct-3 can stimulate the enhancer-dependent transcription in non-EC cells when an appropriate amount of adenovirus E1A protein co-exists. On the basis of their observations, I co-transfected the F1 cells with pCMVOct-3 and an E1A expression vector (pSVE1a), and tried to rescue G418-resistant cells. Two independent clones of G418-resistant cells (RV-1 and RV-2) were successfully rescued from 4 x 10^7 transfected cells.

Southern blots showed that the RV-1 and RV-2 cells had exogenous *Oct-3* and *E1a* genes as well as the 'endogenous' enhancer-trap construct (Figure I-3). This confirmed that the RV-1 and RV-2 cells were indeed derived from the F1 cells. As expected, Oct-3 protein was detected in the RV-1 and RV-2 cells by Western blots (Figure I-6B) and by gel-shift assay (Figure I-5A). Northern blot analysis confirmed the expression of *Oct-3* mRNA as well as E1a mRNA in the RV-1 and RV-2 cells (Figure I-7). The *Oct-3* mRNA detected in the RV-1 and RV-2 cells was larger than the transcript in P19 cells (Figure I-7), indicating that it was the exogenous (not endogenous) *Oct-3* gene which was transcribed in these cells. Both cell lines expressed a lower level of Oct-3 protein and *Oct-3* mRNA than the parental P19 cells, as revealed by Western blot (Figure I-6B) and Northern blot (Figure I-7), respectively.

Importantly, the RV-1 and RV-2 cells showed a phenotype quite different from that of the F1 cells. Since the RV-1 and RV-2 cell lines show a very similar phenotype to each other, I will first describe the properties of the RV-1 cells. First, the RV-1 cells were morphologically distinct; unlike F1 cells, RV-1 cells had round cell body and did not have long processes (Figure I-10). While the F1 cells grew dipersely, the RV-1 cells were more adherent (Figure I-10). The RV-1 cells appeared rather similar to P19 cells in morphology. Furthermore, the RV-1 cells were negative for nestin (Figure I-4) and Brn-2 (Figure I-5A). The most remarkable phenotype of the RV-1 cells was that they could differentiate back to the nestin$^+$/Brn-2$^+$ cells, when Oct-3 expression was lost. RV-1 cells that had been kept in G418-containing medium were transferred to medium lacking G418 and were maintained under non-selective conditions for 7 days. The majority of the cells had lost the expression of the *Oct-3* gene as well as the *E1a* gene (Figure I-7); the cell population that was exposed to the non-selective conditions for 7 days was designated as dRV-1. Most (if not all) of the dRV-1 cells showed a phenotype quite similarly to that of the F1 cells; the dRV-1 cells possessed multiple long processes (Figure I-10), and were positive for nestin (Figure I-4) and Brn-2 (Figure I-5A and B).

Since the dRV-1 cells lacked *Oct-3* function, they were sensitive G418; when they were exposed to G418, the majority of the cells were killed within 7 days. However, G418-resistant colonies appeared, at a frequency of 1 in 2 x 10^3 cells. 5 x 10^2 G418-resistant colonies were pooled and they were designated as rRV-1 ('re-revertants'). The phenotype of rRV-1 cells was indistinguishable from that of RV-1. The rRV-1 cells were morphologically similar to the RV-1 cells (Figure I-10). While the rRV-1 cells had regained the expression of the *Oct-3* and *E1a* genes (Figure I-7), they had lost the expression of Brn-2 (Figure I-5A) and nestin (Figure I-4). Furthermore, the rRV-1 cells could differentiate back to nestin$^+$/Brn-2$^+$ cells again when maintained under the non-selective conditions for 7 days. Such differentiated cells (designated as

drRV-1) lost expression of *Oct-3* and *E1a* genes (Figure I-7), and were positive for Brn-2 (Figure I-5A) and nestin (Figure I-4).

The phenotype of RV-2, another revertant cell line, was similar to that of RV-1. The RV-2 cells were morphologically indistinguishable from RV-1 cells (Figure I-10) and were Oct-3$^+$ and Brn-2$^-$ (Figure I-5A). Furthermore, in phenotypic changes by the absence or presence of G418, the RV-2 series were similar to that of RV-1 (Figure I-5 and 7). The only significant difference between RV-1 and RV-2 was that the RV-2 (and rRV-2) cells needed to be exposed to the medium without G418 for a longer period (2 weeks) until they transformed to the Oct-3$^-$/Brn-2$^+$/nestin$^+$ cells.

Since *E1a* expression also correlated with the phenotypic changes (Figure I-7), we were concerned with the possibility that the phenotypic changes might be simply due to *E1a* expression. To exclude this possibility, the F1 cells were transfected with the E1A expression vector and pSVneo, and were selected with G418. Among hundreds of G418-resistant colonies, none had similar morphology to the RV-1 and RV-2 cells. Twenty colonies were randomly recovered, and several stable transformats expressing *E1a* mRNA were obtained. They were all morphologically indistinguishable from the F1 cells. Two such cell lines (F1/E1a-1 and 2) expressing *E1a* mRNA at a comparable level to the RV-1 cells (Figure I-7) were further examined, and both were Brn- 2$^+$ (Figure I-5). These results now confirmed that the phenotypic changes observed above are not simply due to E1A alone.

The phenotype of various cell lines is summarized in Table I-1. I have also examined SSEA-1, a marker specific to EC cells (Solter and Knowles, 1978). While P19 cells were positive for SSEA-1, RV-1 and RV-2 as well as dRV-1 and dRV-2 were negative. Since my G418-selection described here was based on the transactivating function of Oct-3, I conclude that the gain or loss of the Oct-3 function induced the phenotypic changes from F1 to RV, from RV to dRV, from dRV to rRV, and from rRV to drRV cells.

Discussion

Mechanism of Oct-3 extinction

P19 cells can respond to chemical reagents such as retinoic acid (RA) and DMSO, and differentiate into various cell types (Jones-Villeneuve et al., 1983). *Oct-3* mRNA is extinguished at an early stage of differentiation process. The present study has shown that the fusion to fibroblasts can also initiate differentiation and extinction of *Oct-3* gene expression. Ben-Shushan et al. (1993) also have reported Oct-3 extinction in cell hybrids between F9 EC cells and fibroblasts. The simplest explanation for this phenomenon would be that a 'differentiation-inducing' gene is active in fibroblasts and that this can act dominantly upon the cell fusion. Previous studies utilizing several different hybrid cell systems, among the different phenotypes that appear after fusion, extinction of specific cellular functions (tissue-specific gene expression) are observed (Bergman et al.,1990; Bulla et al., 1992; Junker et al., 1990; MacCormic et al., 1988; Tripputiet et al., 1988). In most cases, it was found that the extinction is accompanied by repression of transactivators that regulate tissue-specific genes. Therefore, repression of *Oct-3* gene may cause the hybrid cell-differentiation.

The expression of the *Oct-3* gene in EC cells is regulated by a stem cell-specific/RA-repressive enhancer (designated as RARE1) located 1 kb upstream of the gene; RARE1 is required for active expression in undifferentiated P19 cells and confers the RA-mediated repression (Okazawa et al., 1991). A role of RARE1 in hybrid cell extinction of the *Oct-3* gene expression is described in Chapter II. In a report of Ben-shushan et al. (1993), it is suggested that the shutdown of *Oct-3* gene expression in RA-differentiated EC cells and in hybrid cells is achieved through changes in the methylation status, chromatin structure, and transcriptional activity of the *Oct-3* gene upstream regulatory region.

I have shown that *Oct-3* gene is extinguished at another level during differentiation; the activity required for the Oct-3-mediated enhancer activation is also lost (Figure I-8 and 9). Therefore, the function of the *Oct-3* gene can be regulated at multiple levels: at the transcriptional level and presumably at the protein-protein interaction level. There may be certain cell types at an early developmental stage in which the Oct-3 protein is still present but cannot function as an enhancer-activator.

Formally, two mechanisms can account for the cell-type specificity of the Oct-3 transactivating domain. One is that the Oct-3 transactivating domain needs to interact with an adapter, which is present only in pluripotent cells such as EC or ES. The other possibility is that the Oct-3 transactivating domain itself is active but differentiated cells possesses a masking factor capable of suppressing the activation domain. In this study, G418-resistant 'revertants' were successfully rescued only when the E1A expression vector was co-transfected. This supports the previous observation by Schöler et al. (1991) that Oct-3 can function as an enhancer-activator in the presence of adenovirus E1A. However, the frequency of the appearance of 'revertants' was extremely low (1 in 10^7 cells). This low frequency may reflect the fact that, in general, an ectopically expressed transcription factor can activate a co-transfected reporter gene but it can rarely activate an endogenous target gene. Alternatively, E1A protein is known to support transactivation by Oct-3 depending on its level; it suppresses the Oct-3 transactivating function when it is present at a high level (Schöler et al., 1991). It is possible that E1A expression was coincidentally at an appropriate level in the RV-1 and RV-2 cells. In any case, it is not certain what role E1A played in rescuing the RV-1 and RV-2 cells—whether E1A protein itself acted as an adapter for Oct-3, E1A induced the adapter, or E1A abolished the interaction with an Oct-3 masking factor.

Role of Oct-3 in EC cell differentiation

In my strategy for selecting cell lines, G418-resistance/sensitivity directly reflects the presence or absence the Oct-3 function. Therefore, any phenotypic differences between the hybrid cells and the revertants (and between RV and dRV cells) must be due to the Oct-3 function. It is not certain by what mechanism the revertant cells lost expression of an exogenous *Oct-3* (and *E1a*) gene when they were exposed to the non-selective conditions (Figure I-7). It is often observed that continuous expression of an exogenous gene requires selection pressure. This is particularly true with P19 cells. In our experience, more than half of pSVneo-transformed P19 cell lines needed to be cultured in the presence of G418 to maintain the active expression of an exogenous gene. An exogenous gene is probably integrated into a previously inactive site of a chromosome. Then, the exogenous gene would remain active only under conditions where its expression was absolutely required for cell growth. When G418 is omitted, the RV-1 cells no longer require expression of the *Oct-3* and *E1a* genes. Alternatively, the presence of the Oct-3 protein or Oct-3 function may be disadvantageous for growth of certain cells such as the hybrid cells. Although, the expression of the *E1a* gene also paralleled the phenotypic changes, our control experiments showed that *E1a* alone was not sufficient to induce the phenotypic changes.

The expression of the Brn-2 and nestin genes was inversely correlated with the expression of the *Oct-3* gene in every cell line studied (Table I-1). This is in good agreement with the behavior of these genes during RA-induced differentiation of P19 cells into neural cells; when P19 cells are treated with RA, the Brn-2 and nestin genes are turned on whereas the *Oct-3* gene is shut off (Fujii and Hamada, 1993). However, it is not certain whether these two genes are under the direct control of Oct-3.

The RV-1 and RV-2 cells resembled P19 cells rather than the F1 cells (Figure II-10). However, there was a difference between RV cells and P19

cells: P19 cells were positive for SSEA-1 whereas RV cells were negative (Table I-1). Therefore, the RV cells are not complete revertants of the F1 cells. Obviously, some of the phenotype of P19 cells were not rescued by the Oct-3 function. It appears that *Oct-3* is not the only gene that specifies the phenotype of pluripotent EC cells.

Oct-3, together with the Oct-3 modulating activity, probably activates a group of genes in the pluripotent cells. However, the target genes of Oct-3 have not been firmly identified yet. In our study, the *neo* gene was driven by the Oct-3-dependent enhancer E1, which was derived from a retrotransposon called 'the early transposon' (Brulet et al., 1983). The perfect correlation between G418 resistance and Oct-3 expression observed among various cell lines (Table I-1) further confirmed that this class of transposon is one of the target genes of Oct-3. Perhaps Oct-3 positively regulates other genes as well, some of which must be activated in the RV cells. The cell lines obtained in this study (such as F1, RV and dRV) may be useful for selecting Oct-3-regulated genes.

In summary, I have shown that P19-L cell hybrids undergo differentiation into neural cells in parallel with the extinction of the *Oct-3* gene expression. Furthermore, the ectopic introduction of Oct-3 function into hybrid cells resulted in dedifferentiation. These observations support the notion that Oct-3 is required for ES cells to maintain pluripotency and that repression of the *Oct-3* gene is required for subsequent differentiation.

Chapter II

RARE1; a stem-cell specific enhancer, acts as a negative regulatory element in differentiated cells

Introduction

Oct-3 gene is expressed in pluripotent stem cells of the pregastrulatory embryo and in germ cells. In situ hybridization studies have revealed that zygotic expression of the*Oct-3* gene starts after the four-cell stage and before 3.5 days postcoitum (dpc). The *Oct-3* gene is repressed subsequently in a lineage-dependent manner. The repression first takes place in the trophectoderm lineage at 4.5 dpc, in the primitive endoderm lineage at 5.5 dpc, in the mesoderm lineage at 7 dpc and finally in the ectoderm lineage at 10 dpc (Rosner et al., 1990; Schöler et al., 1990)

Oct-3 is a good candidate for initial determinant of cell lineages, because it is expressed at the earliest stage of embryogenesis and is repressed at later stages. Moreover, it is suggested that Oct-3 is required for pluripotent stem cells to maintain pluripotency and that repression of the *Oct-3* gene is required for subsequent differentiation (Chapter II). Therefore, studying the regulation of *Oct-3* gene expression may shed light on the mechanisms controlling initial cell-fate determination. Recently, we and others have identified cis-regulatory elements for *Oct-3* gene expression. The *Oct-3* gene is negatively regulated upon RA-induced EC-cell differentiation, by at least two independent cis-acting elements. The cell-type-specific enhancer called RARE1 contributes to the RA-mediated repression, and is located 1.1 kb upstream from the initiation sites (Okazawa et al., 1991). The hormone response element (HRE) in the promoter region is also subject to negative regulation by RA (Pikarsky et al., 1994; Schoorlemmer et al., 1994; Sylvester and Schöler, 1994)

In Chapter I, I have shown that P19-L cell hybrids undergo differentiation into neural cells in parallel with the extinction of the *Oct-3* gene expression. In this study, I analyzed the RARE1 in order to know the mechanism of Oct-3 extinction upon EC cell differentiation. I have found that RARE1 possesses

silencing activity in non-EC cells but not in EC cells. Furthermore, I have identified three independent negative regulatory elements in RARE1, and have detected factors recognizing these elements. In this chapter, we discuss the role of RARE1 in *Oct-3* gene silencing.

Results

RARE1 acts as a negative regulatory element in non-EC cells

In a report by Okazawa et al. (1991), RARE1 was regarded as a stem cell-specific/RA-repressible enhancer in P19 cells. To extend the previous findings, we tested the enhancer activity of RARE1 in other EC cells (F9) and non-EC cells. Unexpectedly, the enhancer was active only in P19 cells, but not in F9 cells (Figure II-1) indicating that RARE1 is not a truly stem cell-specific enhancer. Therefore, we decided to analyzed the activity of RARE1 element from a different point of view. I sought to investigate whether the element can act negatively in non-EC cells. To study the element in this view, I constructed a CAT reporter gene in which the RARE1 fragment was inserted between the SV-40 enhancer and the TATA-box of interferon β gene promoter. A similar assay has been used by others to demonstrate negative activity of other cis-elements (Chou et al., 1991; Keller and Maniatis, 1991; Fujita et al., 1988). As shown in Figure II-2, when this plasmid was transfected into the EC cell lines (P19 and F9), RA-treated P19 cells and non-EC cell lines (L, F1 and HeLa), the RARE1 fragment functioned as a negative element in differentiated cells, but not in EC stem-cells. Among various cell lines examined, there was a perfect reverse correlation between silencing activity of RARE1 and expression of *Oct-3* gene. These results indicate that RARE1 is a negative cis-element which may be involved in repression of *Oct-3* gene .

Mechanism of negative regulation by RARE1

As shown above, RARE1 can block the activity of SV40 enhancer. Although several different mechanisms for negative control of gene transcription are known (Cowell, 1994; Herschbach and Johnson, 1993), two possible mechanisms were considered here. First, RARE1 may act as a silencer which

represses transcription irrespective of its location relative to the enhancer and promoter (Brand et al., 1985). Second, RARE1 may physically block interaction between enhancer and promoter by changing DNA structure or interfering an enhancer binding factor through protein-protein interaction (Levine and Manley, 1989).

To determine the mechanism of negative regulation by RARE1 element in non-EC cells, I constructed several CAT reporters in which the RARE1 was inserted in various positions relative to the SV40 enhancer and TATA-box, transfected these into HeLa cells, and assayed CAT activities (Table II-1). When the RARE1 fragment was inserted between the SV40 enhancer and TATA box, in either orientation, it significantly reduced the activity of SV40 enhancer. On the other hand, when the RARE1 fragment was placed 27 bp upstream from the SV40 enhancer, I could not observe a significant decrease in the SV40 enhancer activity. When the SV40 enhancer was located downstream of CAT gene, insertion of RARE1 upstream of TATA box reduced the activity of SV40 enhancer by about 90% (compare construct 5 with 4). Such position dependence of silencing activity of RARE1 indicates that RARE1 is not a typical silencer, and that it does not interfere with enhancer-promoter interaction. RARE1 may interfere, through RARE1 binding factors, with the activity of some component of the general transcriptional machinery by unknown system different from that of a silencer.

Delineation of RARE1

To localize the negative regulatory element more precisely, I generated CAT reporters in which various shorter fragments of RARE1 were placed between the SV40 enhancer and TATA-box, and transfected them into HeLa cells (Figure II-3). CAT activity from each reporter construct was compared with that from pSVIFNcat. When the RARE1 fragment was divided into two halves at the internal *Alu* I site located at - 1013, both of them (designated as

5' h and 3'h) still had significant silencing activity (approximately 15 fold repression). This indicates that RARE1 contains multiple negative regulatory elements. Therefore, I examined the deletional effect independently in 5'h and 3'h. The results with these deletion mutants indicate that the regions from - 1013 to - 1091 (designated as SE-A) and - 894 to - 936 (designated as SE-B) are most effective, each showed approximately 10-fold repression (Figure II-3). Surprisingly, SE-B was almost identical to RARE1B which is an enhancer component of RARE1. Therefore, I tested RARE1B oligonucleotide (-894 to -935). This fragment also acted efficiently as a negative element (9-fold repression).

HeLa nuclear extracts contains binding activities specific for SE-A and RARE1B

I next searched for binding factors for SE-A and RARE1B by gel shift assay. SE-A binding activity was detected in HeLa nuclear extracts. The specificity of the retarded complex obtained with SE-A probe was established by a series of competition experiments (Figure II-4). Specific competition was obtained with a 200-fold molar excess of unlabeled SE-A fragment, but not with a similar excess of RARE1B oligonucleotide or irrelevant YY1 binding site oligonucleotide. On the other hand, specific binding activity was also detected with RARE1B probe. The retarded complex was competed out by 200-fold molar excess of unlabeled RARE1B oligonucleotide, but not by SE-A fragment and YY1 binding site oligonucleotide (Figure II-4). These results established that the SE-A binding factor is distinct from the RARE1B binding factor.

I further analyzed the SE-A region by DMS interference analysis using the SE-A fragment as a probe. As shown in Figure II-5A, methylation of two purine-rich regions (designated as SE-A1 and 2) partially interferes with binding of the factor. Two interpretations were possible; the same factor

bound to either SE-A1 or SE-A2, or two different factors bound to each sequence.

To distinguish these two possibilities, I next used two oligonucleotides each containing SE-A1 or SE-A2 region (Figure II-5B). In the gel shift assays, a retarded complex observed with SE-A1 was competed out by the unlabeled SE-A1 oligonucleotide, but not by SE-A2 oligonucleotide or irrelevant Sp1 binding site oligonucleotide. On the other hand, two major and one minor retarded complexes were observed with SE-A2, and these were all competed out by the SE-A2 oligonucleotide but not by SE-A1. Moreover, the formation of the complexes observed with SE-A2 was abolished by oligonucleotides containing Sp 1 binding site. These results not only establish that SE-A1 and 2 are recognized by specific yet different factors, but also suggest that the SE-A2 binding factors are members of Sp 1 transcription factor family (Hagen et al., 1992; Imataka et al., 1992; Kingsly and Winoto, 1992). Indeed, the sequence GAGGTGGAG in SE-A2 is very similar to the binding consensus of Sp 1 family (KRGGCKRRK, Faisst and Meyer, 1992).

To assess the functional activity of SE-A1 and SE-A2, each of these oligonucleotide was cloned directly between SV40 enhancer and TATA-box fused to the CAT gene, and was transfected into HeLa cells (Figure II-5C). When the construct containing SE-A1 sequence in either orientation was introduced into HeLa cells, the CAT activity is 5-fold lower than that produced from pSVIFNcat. This level of repression is half of that exhibited by the whole SE-A. On the other hand, SE-A2 alone showed 2-3-fold repression in either orientation. These data indicate that SE-A1 is a major negative element in SE-A, and that SE-A1 and SE-A2 act cooperatively.

Presence of RARE1 binding activities in various cell lines.

The RARE1 binding factors detected in HeLa cells (Figure II-4 and 5b) would be good candidates for differentiation inducing genes. A simple

explanation for the cell-type-specific negative regulation by RARE1 is that repressors recognizing the negative elements exist in differentiated cells, but not in stem cells. To test this possibility, I examined various EC, ES and differentiated cell lines for RARE1 binding factors (Figure II-6).

Specific SE-A1 binding activity was detected in nuclear extracts from all cell lines, and the electrophoretic mobility of the complexes was indistinguishable on the gel. However, the binding activity in EC and ES cells was much lower than that in differentiated cells. Furthermore, the binding activity in P19 cells increased upon RA-induced differentiation. Therefore, the SE-A1 binding factor may be a developmentally regulated repressor.

In the experiment using SE-A2 probe, on the other hand, we could not observe a clear difference between the cell lines tested; three specific complexes were always detected. This result suggests that the same binding factors exist before and after differentiation.

In the case of RARE1B, a binding factor (the upper arrow in Figure II-6) was detected in all the cell lines except for D 2 cells (differentiated P19 cells 2 days after RA-treatment). However, in L, F1 and RV cells, one more retard complex with a higher electrophoretic mobility (the lower arrow) was observed. This suggests that two distinct RARE1B binding factors exist. One is expressed in both stem and differentiated cells and its level is reduced transiently upon RA-induced EC cell differentiation, while the other is L cell specific factor.

Discussion

Mechanism of RARE1-mediated silencing

RARE1 was originally identified as an enhancer controlling the *Oct-3* gene expression in P19 cells(Okazawa et al., 1991). However, RARE1 does not act as an enhancer in F9 cells (Figure II-1). In this study, I have demonstrated that RARE also acts as a negative element with a strict cell-type specificity; it possesses silencing activity in *Oct-3*$^-$ differentiated cells, but not in *Oct-3*$^+$ EC stem cells. These findings suggest that the element actively contributes to the *Oct-3* gene silencing.

RARE1 is not a typical silencer in that its silencing activity is position-dependent (Table II-1). Two different mechanisms have been proposed for position-dependent transcriptional repression (Shen et al., 1994; Herschbach and Johnson, 1993; Nastesan and Gilman 1993). The first is quenching. In the case of c-myc gene silencing, the repressor PRF blocks the function of the activator CF1 by directly contacting the transactivating domain of CF1. PRF can block the activity of CF1 because the PRF binding site is adjacent to, although not overlapping, CF1 binding site upstream of c-myc promoter (Kakkis et al., 1989). In this case, if the repressor binding site was distant from the activator binding site, the transcriptional repression could not be achieved. The cis-element found in the 5'-LTR of Drosophira *gypsy* retrotransposon (Geyer and Corces, 1987; Modolell et al., 1983) can block various enhancers, independently of distance (>100 kb) from the enhancer but only when located between the enhancer and the promoter (Dorsett, 1993; Geyer et al., 1990; Geyer and Corces, 1992; Holdridge and Dorsett, 1991; Jack et al., 1991) . The distance-independent enhancer blocking is achieved by SUHW, the *gypsy* binding protein encoded by *suppresser of Hairy-wing* gene (Holdridge and Dorsett, 1991; Parkhurst et al., 1988). Shen et al (1994) suggest that SUHW increases DNA flexibility and interferes with a protein that

supports long-distance enhancer-promoter interaction. A second mechanism is interference of enhancer-promoter interaction by structural change of DNA between the enhancer and the promoter. YY1 is a ubiquitously expressed DNA binding protein that possesses GLI-kruppel type zinc-finger motif. It either represses or activates transcription depending on the promoter context(Hahn, 1992). Nastesan and Gilman (1993) have shown that YY1 blocks the activity of cyclic AMP responsive element (CRE) on c-fos gene promoter by DNA bending between CRE and TATA box. The repression by YY1 is dependent on the orientation of the two YY1 binding sites.

The negative regulatory activity of RARE1 is not analogous to any of those described above, since RARE1 acts negatively for the gene transcription even if the enhancer is located downstream of the transcription start. In order to determine the mechanism of RARE1 mediated transcriptional repression, cloning and analysis of RARE1 binding repressors are required.

RARE1 binding factors

RARE1 contains three independent negative elements. Deletional analysis (Figure II-3) indicates that each element alone is not sufficient for full activity of RARE1. Therefore, these elements appear to function cooperatively.

RARE1B is an enhancer component of RARE1, and yet can act as a negative regulatory element in differentiated cells. Furthermore, HeLa nuclear extracts contain a specific RARE1B binding factor. The RARE1B binding activity was detected in both stem (EC and ES) cells and differentiated cells. However, it disappeared transiently upon RA-induced differentiation of P19 cells. The transient disappearance of the binding activity suggests that the binding activity is not involved in the initiation of *Oct-3* gene silencing while it may be required for maintaining *Oct-3* gene off. It is not clear why RARE1B acts as a negative element in differentiated cells whereas no significant change is observed with the RARE1B binding activity upon EC cell differentiation.

Three mechanisms could account for this phenomenon. First, there may be two distinct binding factors with the same electrophoretic mobility each of which has opposite activity (activation or repression). Second, the RARE1B binding factor may be is modified (such as phosphorylation and glycocylation) in differentiated cells. Upon modification, the factor may lose the transactivating activity and gain the repressing activity. Finally, additional factors expressed in a cell-type-specific fashion may be required for the transactivating or repressing activity. Several instances that one protein has both activating and repressing activity have been reported previously (Herschbach and Johnson, 1993; Cowell, 1994). In some of these cases, additional factors are required for the function. On the other hand, the Drosophila *kruppel* gene product can act either as a transcriptional activator or as a repressor depending on its concentration (Säver and Jackle, 1993). For understanding of the RARE1B mediated repression of *Oct-3* gene, cloning of the RARE1B binding factor is required.

SE-A1 is also a differentiated cell-specific negative regulatory element (Figure II-5 and 6). A SE-A1 specific binding activity identified in HeLa cells was also detected in P19 stem-cells at a lower level, and increased RA-responsibly to a similar level of that in HeLa and other non-EC cell lines. The correlation between the silencing activity and the binding activity suggests that the SE-A1 binding factor is a developmentally regulated transcriptional repressor and contributes to initiation and maintenance of *Oct-3* gene silencing. I have not been able to find a negative element identical to SE-A1 (TGGAGGAAGGGAAG). However, binding sequences of some transcription factors are similar to the SE-A1 site. The consensus binding sequences of *ets* protoncogene family products; MMGGAWRY (Faisst and Meyer, 1992) is most similar. MAZ/Pur 1, a transcriptional activator regulating c-myc and insulin gene expression, also binds to purine-rich sequences (GGGAGGG/AGAGAGGAGGTG) related to the SE-A1 site (Bossone et al,

1992; Kennedy and Rutter, 1992). It is unknown whether the SE-A1 binding factor is related to any of these factors.

The silencing activity of SE-A2 is lower than that of SE-A1. However, SE-A containing both A1 and A2 can act more effectively as negative element than A1 or A2 alone. These observations suggest that SE-A2 itself is not so sufficient for the cell-type-specific silencing activity of RARE1, yet it amplifies the activity of SE-A1. The results from gel-shift assays (Figure II-5B and 6) suggest that the SE-A2 binding proteins probably belong to Sp1 family. First, the three specific complexes detected in HeLa nuclear extracts were competed out by Sp1 binding site. Second, the gel-shift pattern (Figure II-5B) is very similar to that of Sp1 and Sp3 (a member of Sp1 family; Hagen et al., 1992 and 1994). Furthermore, Sp1 and Sp3 are expressed ubiquitously like SE-A2 binding factors (Hagen et al., 1992).

In summary, I have demonstrated that RARE1 contains multiple negative elements that potentially contribute to the *Oct-3* gene silencing. However, RARE1 is not a typical silencer, and its silencing activity is position dependent. Under the situation where positive regulatory elements of *Oct-3* gene have not been fully understood, it is not clear to what extent RARE1 contributes to the *Oct-3* gene silencing. To know the role of RARE1 in the *Oct-3* gene regulation more precisely, it is necessary to identify the RARE1 binding factors at a molecular level.

Abstract

Abstract

The *Oct-3* gene is expressed in highly undifferentiated cells and is implicated in mammalian early embryogenesis. We have generated a series of hybrid cells between pluripotent embryonal carcinoma cells (Oct-3$^+$) and fibroblasts (Oct-3$^-$), and have studied the regulation and function of *Oct-3*. Upon fusion, the hybrid cells differentiated to nestin$^+$/Brn-2$^+$ cells resembling neuroepithelial stem cells. Expression of *Oct-3* was extinguished at the transcriptional level in all the hybrid cells examined. The Oct-3 modulating activity required for the Oct-3-mediated enhancer activation was also extinguished. When the Oct-3 transactivating function was introduced into the hybrid cells, they transformed into morphologically distinct nestin$^-$/Brn-2$^-$ cells ('revertant'). When the 'revertant' cells subsequently lost *Oct-3* expression, they differentiated back to nestin$^+$/Brn-2$^+$ cells. The close correlation between the phenotypic changes and the gain/loss of Oct-3 function indicates that Oct-3 can induce dedifferentiation of the neural cells. These results strongly suggest that Oct-3 is required for maintaining pluripotency of early embryonic cells and that the repression of the *Oct-3* gene is required for subsequent differentiation.

In order to know the mechanism of the *Oct-3* gene silencing, we have analyzed a stem cell-specific/RA-repressive enhancer (RARE1) of *Oct-3* gene. We have found that RARE1 also possesses silencing activity in differentiated cells depending on its position relative to the enhancer and promoter. Deletional analysis, gel shift assay and DMS-interference assay showed that the silencing activity of RARE1 is achieved by at least three independent elements, each of which is recognized by distinct factors. These factors may be involved in stem cell differentiation by silencing the *Oct-3* gene.

Materials & Methods

Materials & Methods

DNA constructions

DNA sequence, and construction of addition and deletion constructs were carried out by standard molecular biological procedure (Sambrook et al., 1989).

pCMVOct-3 was constructed by inserting the full-length Oct-3 cDNA (*Xba -Xho*) fragment into the corresponding site of an expression vector driven by the CMV promoter. The E1A expression vector (referred to as pSVE1a: Shiroki and Toth, 1988) was kindly provided by K. Shiroki and H. Kato. The GAL4/Oct-3 expression vector constructed as described below. The Oct-3 cording region (codons 1-134) was obtained by PCR as a *Xba - BamHI* fragment. This fragment was subcloned in-frame into the corresponding site of the CMV promoter-driven expression vector in such a way that the Oct-3 transactivating domain is fused to the carboxyl-terminus of the GAL4 DNA binding domain (codons 1-147). GAL4/VP16 expression vector was kindly provided by M. Ptashne (Sadowski et al, 1988). A reporter plasmid containing a GAL4 binding site (1xUAS - CAT; Yoshimura et al., 1989) was kindly provided by Drs J. Fujisawa and M. Yoshida. pIFNcat (Hata et al., 1989) was kindly provided by Drs S.Ono and A. Hata. pIFNcat contains a human β-interferon gene promoter fragment (-55 to +19) including the TATA box. pSVIFNcat and pIFNcatSV were constructed as described below.The SV40 enhancer (*Pvu* II - *Bam* HI fragment from pSV2CAT) was cloned at the *Sma* I - *Bam* HI site of pUC12(designated as pE^{SV}), and then, *Bam* HI smaller fragment of pIFNcat was inserted into the *Bam* HI site in either orientation (pSVIFNcat and pIFNcatSV). To generate SV- RARE1-IFNcat fusion genes (see Figure III-3), the RARE1 region (nucleotide -1132 to -889) obtained by PCR as a *Eco* RI fragment was subcloned at corresponding site of pBluescript (pRARE1). The *Sma* I - *Eco* RV smaller fragment of pRARE1 was inserted

into *Sma* I site between SV40 enhancer and IFN promorter of pSVIFNcat in either orientation and into *Sma* I site upstream of IFN promorter of pIFNcatSV in the same orientation as in the Oct-3 gene(pRARE1catSV). To place RARE1 5' to the SV40 Enhancer, the *Eco* RI smaller fragment of pRARE1 was inserted into the corresponding site of pE^{SV}, and a clone with single copy of the RARE1 in the same orientation as in the pSVRARE1cat was identified, finally, IFNcat fragment was inserted as same way as construction of pSVIFNcat (pRARE1SVcat).

Deletion constructs of pSVRARE1cat plasmid were generated as described below. The ΔA-RARE1 fragment (Okazawa et al., 1991) subcloned in *Xba* I - *Bam* HI site of pBluescript was first linearized with *Bam* HI - *Pst* I (for 3' deletion) or *Sac* II - *Xba* I (for 5' deletion) followed by digestion with exonuclease III (Takara) for up to 300 sec at 25°C. Aliquots were taken at 5 min intervals at 65°C, added into a solution containing mung bean nuclease (Takara), and incubated for 1hr at 37°C. Following transformation of this reaction, the series of plasmids with appropriately sized inserts were chosend, and cleaved with *Eco* RV - *Xba* I (3'- deletion mutants) or *Sac* I - *Eco* RV (5'- deletion mutants). The *Xba* I - *Eco* RV fragments were cleaved with *Alu* I to cut out the 5'- half of RARE1 fragment. The *Sac* I - *Eco* RV fragments were blunted by using the T4 DNA polymelase(Toyobo) and cleaved with *Alu* I to cut out the 3'- half of RARE1 fragment. Finally, these fragments were all inserted into *Sma* I site of pSVIFNcat, and the sequence and orientation were confirmed by sequencing.

Oligonucleotide

The oligonucleotides used for DNA-binding assays are as follows(show only cording strand): OCTA26, 5' - GATCAGTACTAATTAGCATTATAAAG - 3'; RARE1B, 5' - AGCCATCCTGGCCCATTCAAGGGTTGAGTACT - 3'; YY1, 5' - AGGGTCTCCATTTTGAAGCGGG - 3'; Sp1, 5' - TCGAACGGG GCGGGGCG - 3'; SE-A1, 5' - TCCCTGGAGGAAGGGAAGCAGGGTATC

T - 3'; SE-A2, 5' - GTCTTTGAGGAGAGGTGGAGAGCTGGG - 3'.

Cell culture and differentiation

The P19, F9, PCC3, L and HeLa cell lines were grown in α-MEM medium supplemented with 10% fetal bovine serum.

Cell differentiation of P19 cells was induced as follows. The cells were plated in bacterial-grade dishes in the presence of 1 μM RA. The cell aggregates thus formed were plated on tissue culture-grade dishes on 4 days after the induction. ES cells established from strain SV129SVJ mice were routinely maintained(Azuma and Toyoda, 1991) in the absence of feeder cells in medium supplemented with murine DIA/LIF.

Isolation of the hybrid cells and their derivative cell lines

052, a P19 cell line containing a single copy of the enhancer-trap, has previously been described by Bhat et al (1988). An HGPRT-deficient 052 cell line was isolated by selecting 052 cells in the presence of 10 μM 6-thioguanine, as described by Hooper (1987). Cell fusion was performed essentially as described by Hooper (1987). In brief, equal numbers of HGPRT-deficient 052 cells and TK-deficient L cells were plated on 10 cm dishes, and cell fusion was induced with polyethylene glycol. The cells were trypsinized, serially diluted and plated again. The hybrid cells were selected with HAT medium lacking G418. HAT-resistant colonies appeared at a frequency of 1 in 10^2 cells and were indistinguishable in morphology. 20 colonies were recovered, propagated and established as independent cell lines (F1 - F20). They were stable over several passages; at least, F1 has been passaged more than 10 times without phenotypic changes. All the hybrid cell lines were sensitive to G418 (200μg/ml). To isolate 'revertants', one of the hybrid cell lines (F1) was co-transfected with the Oct-3 expression vector (pCMVOct-3) and the E1A expression vector (pSVE1a; Shiroki and Toth, 1988) at various ratios. The

transfected cells were exposed to 200μg/ml of G418. One G418-resistant colony (RV-1) appeared on a dish that received 10 μg of pCMVOct-3 and 5μg of pSVE1a. Another G418-resistant colony (RV-2) was obtained in a separate transfection experiment. The G418-resistant colonies were recovered, and were propagated in the G418- containing medium. To obtain dRV-1, the RV-1 cells were transferred to lacking G418 and were maintained in the absence of G418 for 7days. A whole cell population that was exposed for the non-selective condition for 7days was designated as dRV-1. The dRV-1 cells were maintained in the absence of G418. To obtain rRV-1, the dRV-1 cells were returned to the medium containing 200μg/ml of G418. Although most of the dRV-1 cells were killed within 7days, G418 resistant colonies appeared at a frequency of 1 in 2 x 10^3. About 5 x 10^2 G418-resistant colonies pooled and maintained in the presence of G418 (designated as rRV-1). The drRV-1 cells were isolated from rRV-1 cells, as dRV-1 was obtained from RV-1. A series of sublines of RV-2 (dRV-2, rRV-2 and drRV-2) was obtained from RV-2 in a similar way, except that RV-2 and rRV-2 were exposed to the G418-free medium for a longer period (2 weeks) in order to convert them dRV-2 and drRV-2, respectively. Unless otherwise mentiond in the absence of G418, whereas 052, HGPRT-deficient 052, RV-1, rRV-1, RV-2 and rRV-2 were maintained in the presence of 200 μg/ml G418.

To isolate F1 cells expressed E1A, the F1 cells were transfected with pSVE1a and pSVneo and were selected with 200 μg/ml G418. All the G418-resistant colonies were morphologically indistinguishable from the F1 cells. Twenty G418-resistant colonies were recovered and established as cell lines. In eight of them, *E1a* mRNA was detected in Northern blot. Two such cell lines (F1/E1a and 2), expressing *E1a* mRNA at a comparable level to the RV-1 cells, used as controls in this study.

Blot hybridizations

Cellular RNA and DNA isolation, and blot hybridizations were carried out

by standard techniques (Sambrook et al., 1989). Each DNA probe for hybridization was labeled with using Randam Prime Labelling Kit (Boheringer).

Transfections and CAT assay

Transfections were essentially as described by Chen and Okayama (1984). For transfection into RA-treated P19 cells, 1μM RA was added when the cells were plated. RA was presented until the cells were harvested. Therefore, the cells were exposed to RA for 48 hr. CAT assays were done as described by Gorman et al (1982). The CAT activity was normalized to equivalent β-galactosidase activity from the expression vector pCH110 included as an internal standard, and quantitated on a Image Analyzer BAS 2000 (Fuji film).

Antibodies

For the antibody against Oct-3, a GST/Oct-3 chimeric protein in which the amino-terminal part of Oct-3 (residues 1 - 134) was fused to glutathione S-transferase (GST), was produced in *Escherichia coli*. The fusion protein was purified by glutathion-Sepharose chromatography and injected into a rabbit. The rabbit antiserum was adsorbed with GST protein, affinity purified on GST/Oct-3-coupled Sepharose and used for Western blot was analysis at 1/1000 dilution. Western blot was performed with an alkaline phosphatase-conjugated goat anti-rabbit IgG antibody. The anti-nestin antibody was raised in rabbit with bacterially synthesized nestin protein as a antigen (Lendhal et al., 1990). For histochemical staining of nestin, cells were fixed with 4 % paraformaldehyde. The nestin antibody was used at 1/1000 dilution. A fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG antibody was used as the second antibody.

Preparation of nuclear extracts and DNA-binding assays

Nuclear extracts were prepared essentially as described by Schreiber et al.,

(1990) with minor modification. Briefly, nuclei were extracted with high salt buffer [20 mM HEPES-KOH (pH7.9), 0.4 M NaCl, 20% (vol/vol) glycerol, 1.5 mM $MgCl_2$, 0.1 mM EDTA, 0.1mM EGTA, 1mM DTT, 1mM PMSF, 1mM Benzamidine, 1μg/ml Antipain, 1μg/ml pepstatin A, 1μg/ml leupeptin] and, after centrifugation at 10,000 x g for 20 min, the supernatant was collected and stored at - 80 °C.

All gel shift assays were performed with 20 μl reaction that contained 40 mM HEPES-KOH (pH 7.9), 80 mM NaCl, 15% (vol/vol) glycerol, 0.3 mM $MgCl_2$, 1 mM EDTA, 0.02 mM EGTA, 2 μg of poly [d(I-C)], 0.2 ng of end labeled double strand DNA fragment and 8~16 μg of nuclear protein. After incubation for 30 min at 22 °C, the reaction mixtures were loaded on pre-run 4% polyacrylamide gel (29:1) containing 1x TAE, and electrophoresed for 2 hr at 125 V. The gels were fixed, dried and autoradiographed. For the super-shift assay, the anti-Brn-2 antibody was raised in a rabbit with the GST/Brn-2 fusion protein as an antigen (Fujii and Hamada, 1993). The antibody was purified by passing it through a GST-Sepharose column followed by binding to protein A-Sepharose.

DMS interference assay was done essentially as described by Baldwin et al. (1988). Briefly, after the 10 - 30 fold scale of gel shift used the single end-labeled probe modified with DMS, the protein-DNA complex and the free probe were excised from the gel, eluted, ethanol precipitated and cleaved with 1 M piperidine. These samples were then analyzed on an 8 M urea/10% polyacrylamide sequencing gel.

Acknowledgments

Acknowledgments

The author wishes to express profound thanks to Dr. Hiroshi Hamada (The Tokyo Metropolitan Institute of Medical Science), Prof. Masami Muramatsu (Saitama medical collage) and Prof. Hiroto Okayama (Tokyo University) for their valuable guidance in the course of this study and critical reading of the manuscript, Drs. Hitoshi Okazawa, Hideta Fujii and Ronald D.G. Mckay for their collaboration.

The author also wishes to thank M. Ikeda and K. Tamai for production of antibodies against Oct-3 and Brn-2, Drs T. Muramatsu and D. Solter for the antibody against SSEA-1, Dr. M. Tsuda for the advice on DNA-binding assay, M. Okuda-Noda and Dr. Y. Maeda for oligonucleotides synthesis, Dr. M. Imagawa for preparation of the antigen for Oct-3 antibody, Drs. J. Fujisawa and M. Yoshida for the Gal4 reporter plasmid, and Dr. M. Ptashne for the Gal4/VP16 plasmid.

The author also wishes to thank our companions in Department of Biochemistry, Faculty of Medicine, University of Tokyo and in Department of Cancer Therapeutics, The Tokyo Metropolitan Institute of Medical Science for their kind and helpful advise during the course of this study.

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Figures & Tables

Figure I-1. *Experimental strategy for selecting the hybrid cells and their derivative cell lines.* The strategy for selecting the hybrid cell lines and their derivatives is summarized. Cell line 052 is transformed with one copy of an enhancer-trap, an enhancerless plasmid with the *neo* gene (Bhat et al., 1988). The trap is integrated near the endogenous gene is driven by the transposon-derived promoter (open circle) and the Oct-3-dependent enhancer E1 (closed circle). 6-TG, 6-thioguanine. See text for details.

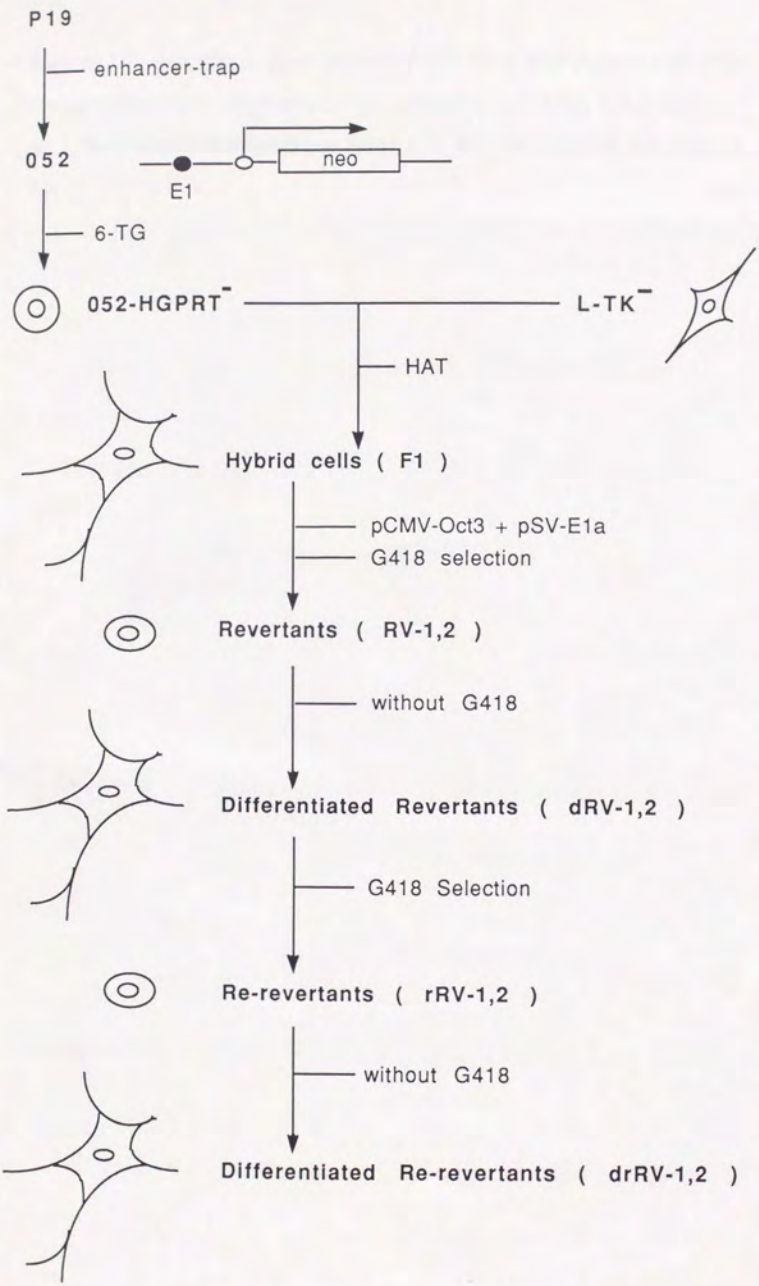

P19
enhancer-trap
052
neo
E1
6-TG
052-HGPRT⁻
L-TK⁻
HAT
Hybrid cells (F1)
pCMV-Oct3 + pSV-E1a
G418 selection
Revertants (RV-1,2)
without G418
Differentiated Revertants (dRV-1,2)
G418 Selection
Re-revertants (rRV-1,2)
without G418
Differentiated Re-revertants (drRV-1,2)

Figure I-2. *Morphology of parental cell lines and hybrid cell lines.* Phase contrast photographs of two parental cell lines (052HGPRT$^-$ and L-TK$^-$) and four hybrid cell lines (F1, F2, F3 and F4) are shown.

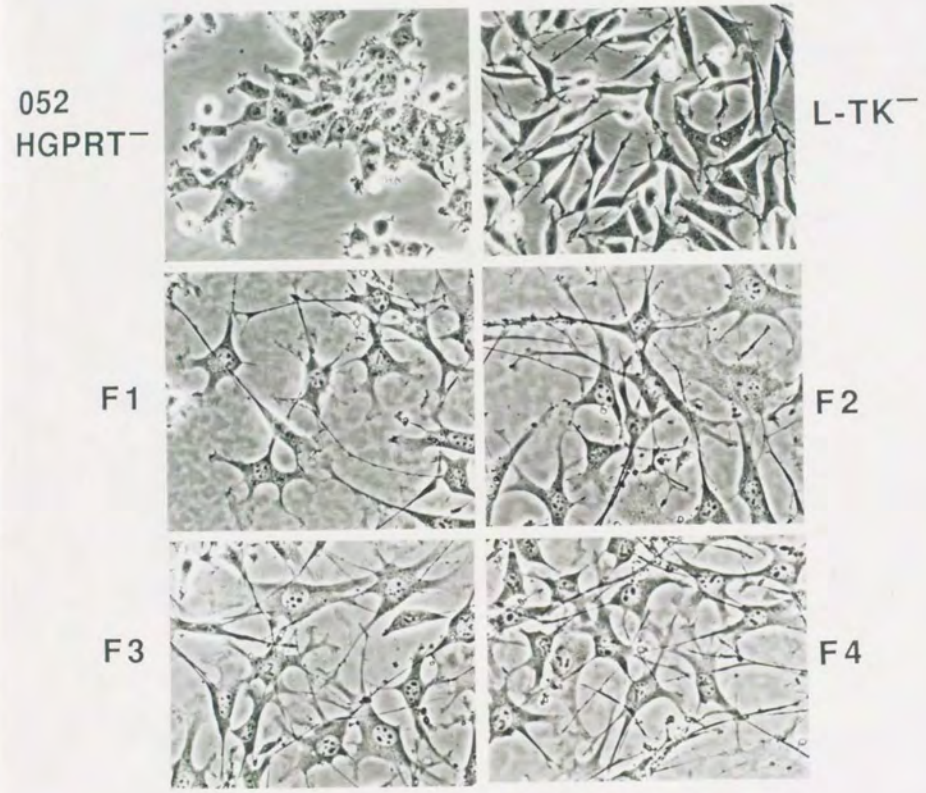

052
HGPRT⁻
L-TK⁻
F1
F2
F3
F4

Figure I-3. *The enhancer-trap copy is stably maintained during the cell fusion and G418 selection.* Genomic DNA from the indicated cells was digested with *Eco* RI and subjected to Southern blot. Three probes were used; the enhancer-trap plasmid (pA_{10}neo; Bhat et al., 1988), *Oct-3* and *E1a*. Since the whole pA_{10}neo plasmid was used as a probe in the 'pA_{10}neo' panel, this probe detected the DNA fragments derived from the enhancer-trap (open circles) as well as fragments derived from exogenously introduced pCMVOct-3 and pSVE1a (closed circles). In the 'Oct-3' panel, open circles show the endogenous *Oct-3* gene, closed circles show exogenously introduced *Oct-3* gene, and (X) shows endogenous *Oct-3* related genes.

pA10 neo
F1
F2
F3
F4
F5
L-TK−
052 HGPRT−
052
RV-1
dRV-1
rRV-1
drRV-1
RV-2
Oct-3
052
F1
RV-1
dRV-1
rRV-1
drRV-1
X
E1a
052
F1
RV-1
dRV-1
rRV-1
drRV-1

Figure I-4. *Inverse correlation between Oct-3 and nestin expression.* The indicated cells were fixed with 4% paraformaldehyde and incubated with an anti-nestin antibody. Nestin was visualized using an FITC-conjugated second antibody.

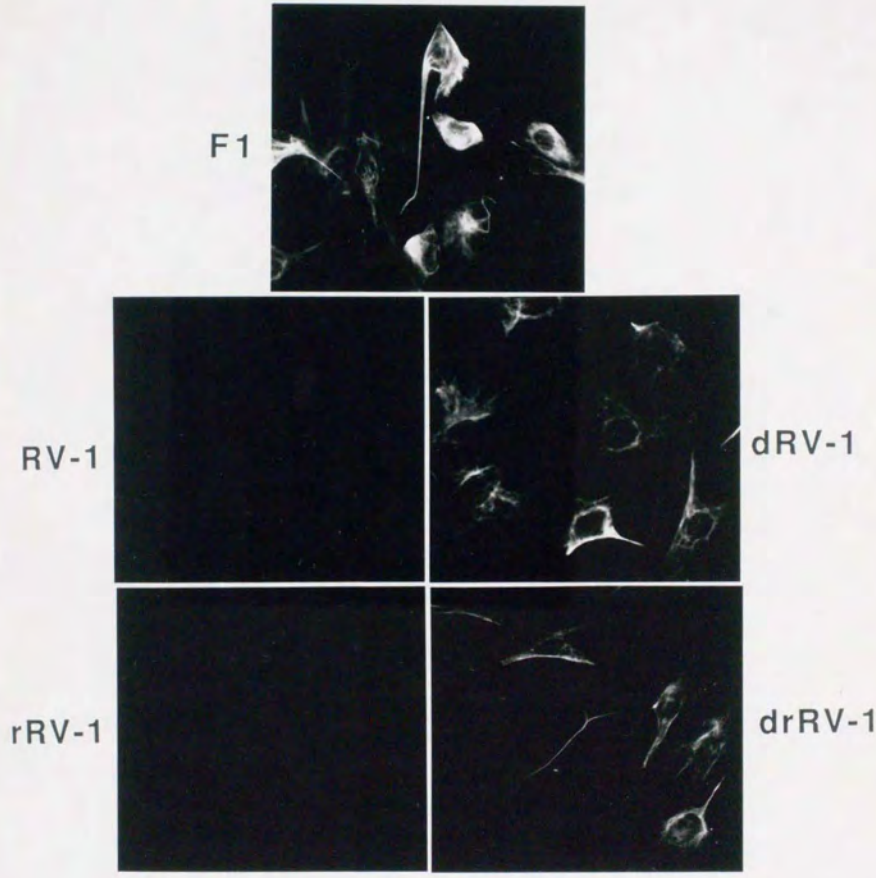
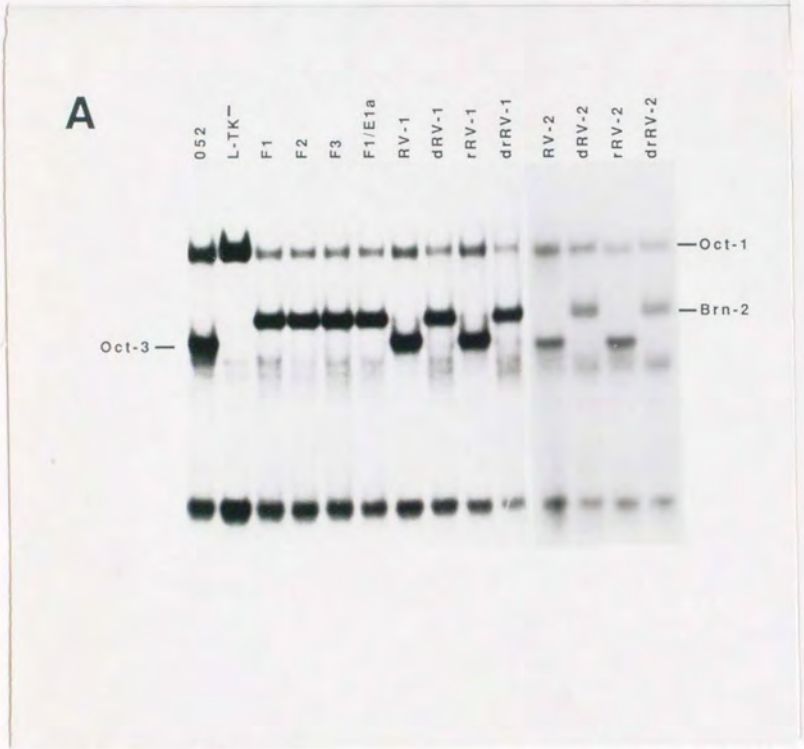

F1
RV-1
dRV-1
rRV-1
drRV-1

Figure I-5. *Inverse correlation between Oct-3 and Brn-2 expression.* (A) Nuclear extracts were prepared from the indicated cells. The octamer-binding activity in each extract was examined by gel shift assay. Three octamer-binding factors (Oct-1, Oct-3 and Brn-2) are indicated. Note that the expression of Oct-3 and Brn-2 is inversely correlated. (B) Nuclear extracts from the F1 and dRV-1 cells were subjected to the binding assay in the presence of the anti-Brn-2 antibody or anti-Oct-3 antibody. The super-shifted bands containing Brn-2 are indicated by the closed circle.


A
052
L-TK⁻
F1
F2
F3
F1/E1a
RV-1
dRV-1
rRV-1
drRV-1
RV-2
dRV-2
rRV-2
drRV-2
Oct-3
Oct-1
Brn-2

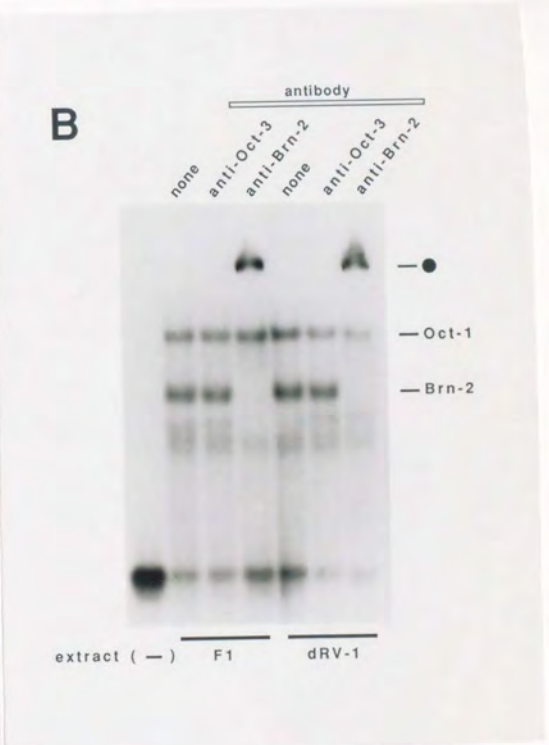

B
antibody
none
anti-Oct-3
anti-Brn-2
none
anti-Oct-3
anti-Brn-2
Oct-1
Brn-2
extract (—)
F1
dRV-1

Figure I-6. *The level of Oct-3 protein in various cells.* **(A)** P19 cells were induced to differentiate with RA. Cell lysates were prepared at indicated time after the induction, and Oct-3 protein level was assayed by Western blot analysis. The 43 kDa protein (Oct-3) is indicated by the arrow. Note that the 43 kDa protein was detected in P19 cells but disappeared 24 h after the induction. **(B)** Cell lysates were also prepared from P19 cells (D^-), P19 cells treated with RA for 8 days (D^+), the hybrid F1 cells and RV-1 cells. The Oct-3 level was determined by Western blot analysis.


R A
(kd) M 0 0.5 1 2 3 4 5 6 7 8 (days)
97
69
46
30
A
P19
D- D+ F1 RV-1
B
Oct-3

Figure I-7. *Northern blot analysis of Oct-3 and E1a mRNA.* 20 μg of cytoplasmic RNA from various cell lines were subjected to Northern blot analysis. For RV-1 and rRV-1, two different batches of RNA were examined. Note that *Oct-3* mRNA expressed in the RV-1, rRV-1, RV-2 and rRV-2 cells is apparently larger than endogenous *Oct-3* mRNA present in P19 (052) cells.

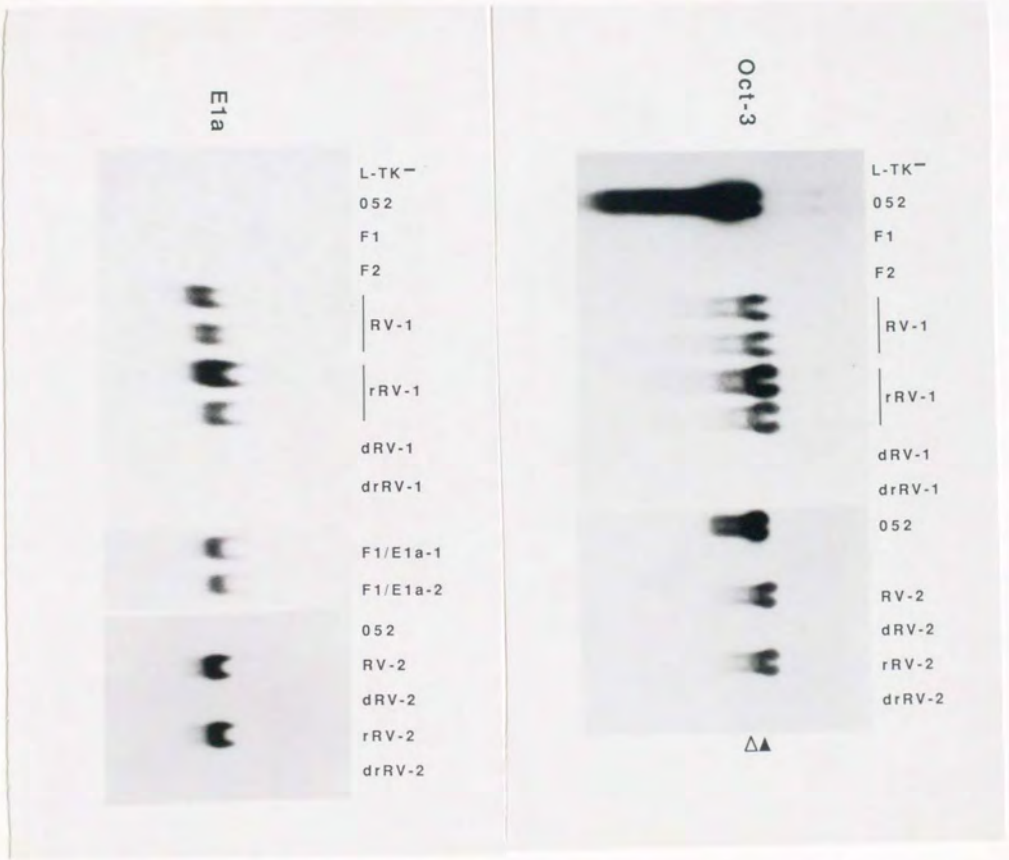

E1a
L-TK⁻
052
F1
F2
RV-1
rRV-1
dRV-1
drRV-1
F1/E1a-1
F1/E1a-2
052
RV-2
dRV-2
rRV-2
drRV-2
Oct-3
L-TK⁻
052
F1
F2
RV-1
rRV-1
dRV-1
drRV-1
052
RV-2
dRV-2
rRV-2
drRV-2
△▲

Figure I-8. *Oct-3 alone cannot stimulate the enhancer-dependent transcription in differentiated cells.* Structure of two reporter CAT plasmids are shown on top. pOCTAcat (a) contains six tandem copies of octamer-motif sequences (closed circles) linked to the enhancerless SV40 early promoter (open circle). The other reporter, pBScat (b) lack the octamer-motif sequences. In lanes 1-7 and 10-14, one of the reporter plasmids was transfected into indicated cells, with or without the Oct-3 expression vector (pCMVOct-3). In lanes 5-7 and 11-14, an increasing amount (2,4 and 8 μg) of pCMVOct-3 was transfected. pCH110 was included as an internal standard. CAT assay was performed as described in Material and methods. In lanes 8 and 9, Oct-3 protein produced in HeLa cells was analyzed by Western blot. Lane 8, cell lysates from HeLa cells transfected with pCMVOct-3; lane 9, cell lysates from untransfected HeLa cells. The 43 kDa protein (Oct-3) produced by the expression vector is shown by the arrow head.

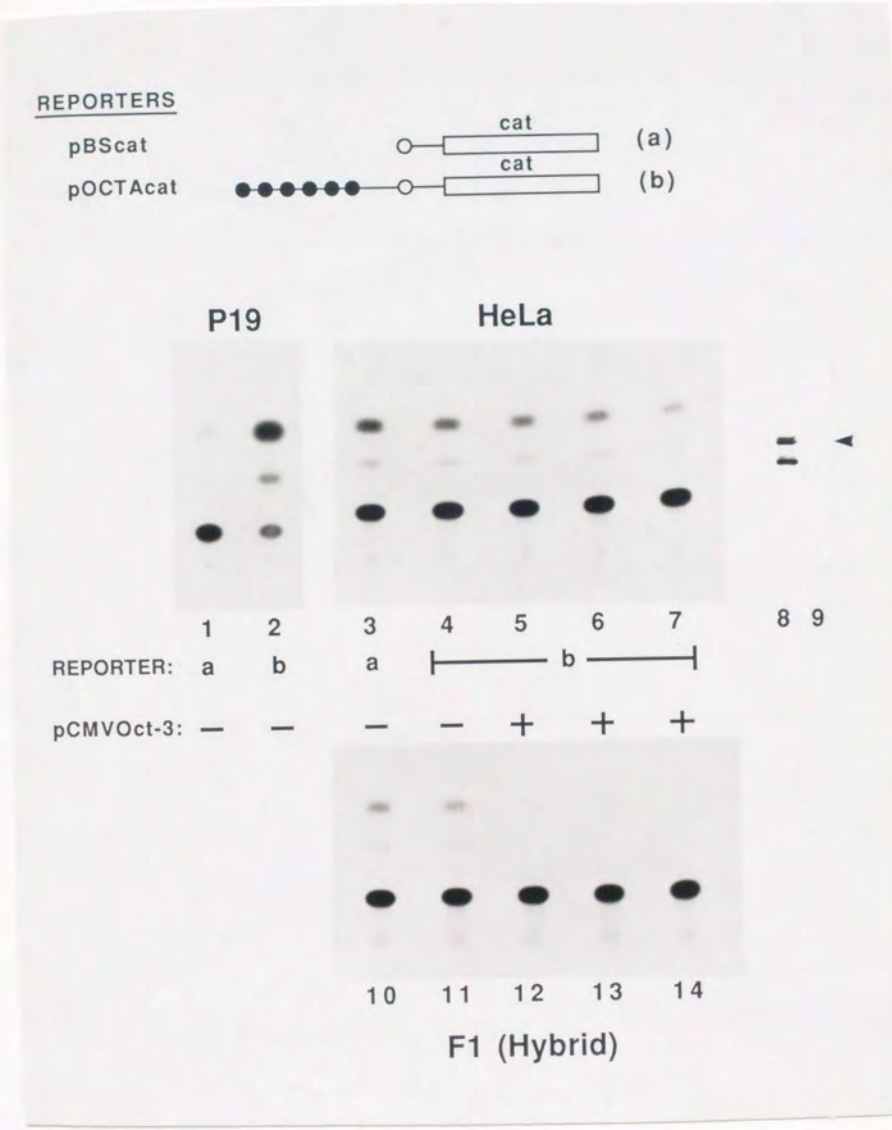

REPORTERS
pBScat
pOCTAcat
cat
cat
(a)
(b)
P19
HeLa
1 2 3 4 5 6 7 8 9
REPORTER: a b a b
pCMVOct-3: — — — — + + +
10 11 12 13 14
F1 (Hybrid)

Figure I-9. *The transactivating domain of Oct-3 functions in a strictly cell-type-specific fashion.* 10 μg of reporter (1xUAScat) and 5 μg of one of the effectors were transfected into P19 cells, the F1 cells or L-TK$^-$ cells. An equivalent amount of cell lysate was assayed for CAT activity. Structures of the reporter and effectors are shown on the top. Open circle, a promoter from HTLV-LTR; closed circle, a binding site for GAL4; closed rectangle, the GAL4 DNA-binding domain; shaded rectangle, the Oct-3 transactivating domain; open rectangle, the transactivating domain derived from HSV-VP16. Note that ΔGal4 contains only the Gal4 DNA-binding domain.

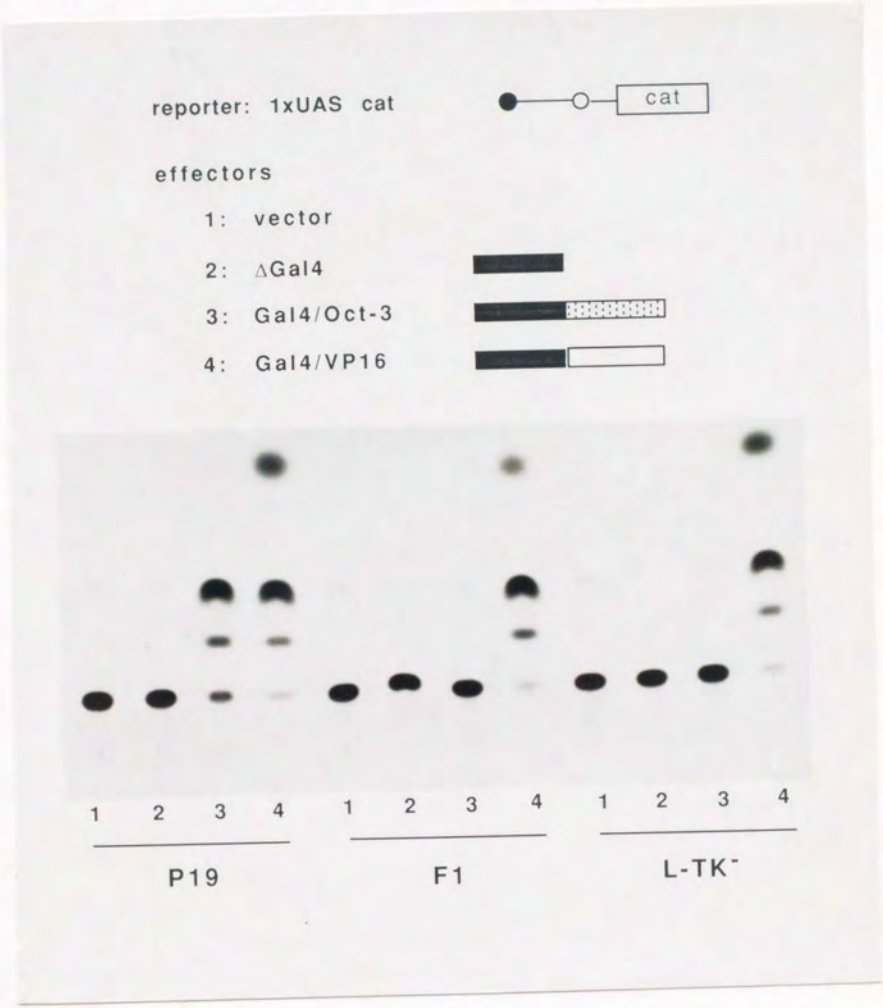

reporter: 1xUAS cat
cat
effectors
1: vector
2: ΔGal4
3: Gal4/Oct-3
4: Gal4/VP16
1 2 3 4 1 2 3 4 1 2 3 4
P19
F1
L-TK-

Figure I-10. *Morphplogy of two 'revertant' cell lines.* Phase contrast photographs of twe 'revertant' cell lines (RV-1 and RV-2) and their derivative cell lines are shown. See text for details.

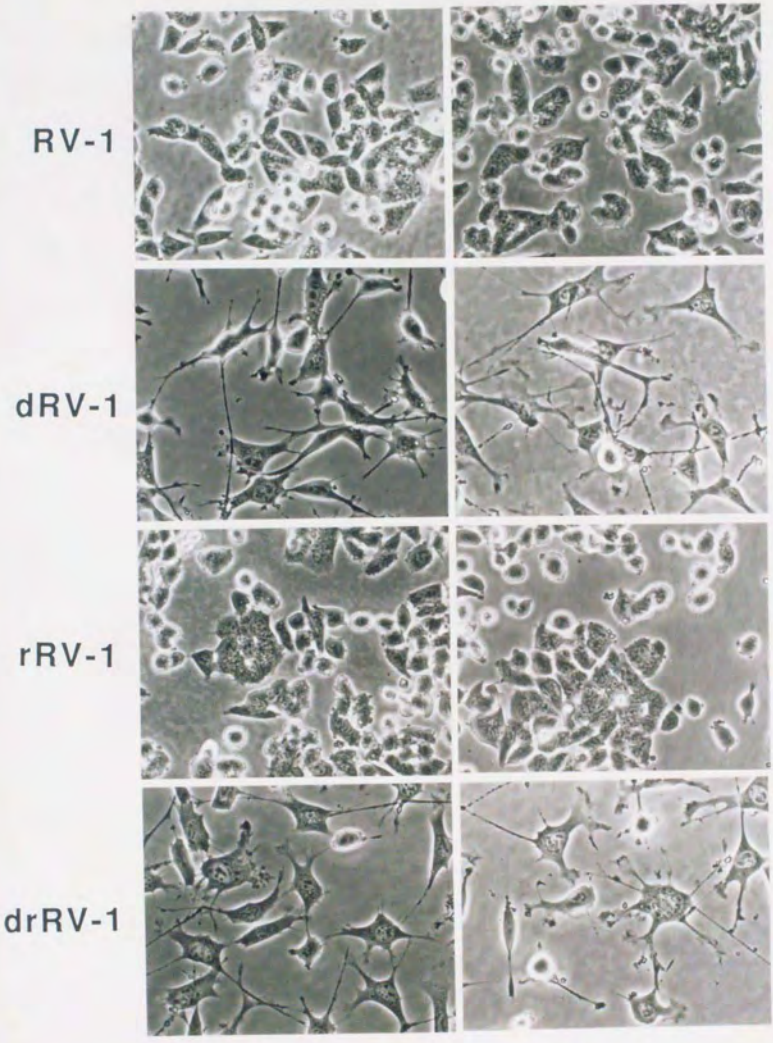

RV-1
RV-2
dRV-1
dRV-2
rRV-1
rRV-2
drRV-1
drRV-2

Figure II-1. *The enhancer activity of RARE1 is P19 cell specific.* The structure of the CAT reporter constructs are shown on the top. a: β-IFN gene TATA box (from -55 to +19) was fused to the CAT gene. b: The RARE1 containing 243 bp fragment (-1132 to -889) was placed 45 bp upstream from the TATA box. c: The SV40 enhancer (Pvu II - Bam HI) fragment from pSV2CAT was placed 50 bp upstream from the TATA box. Typically, 10 μg of reporter plasmid, 2 μg of pCH110 as a internal standard, and 13 μg of pBluescript as a carrier DNA were transfected into indicated cell line(5×10^6 cells/10 cm dish) and assayed for the CAT activity.

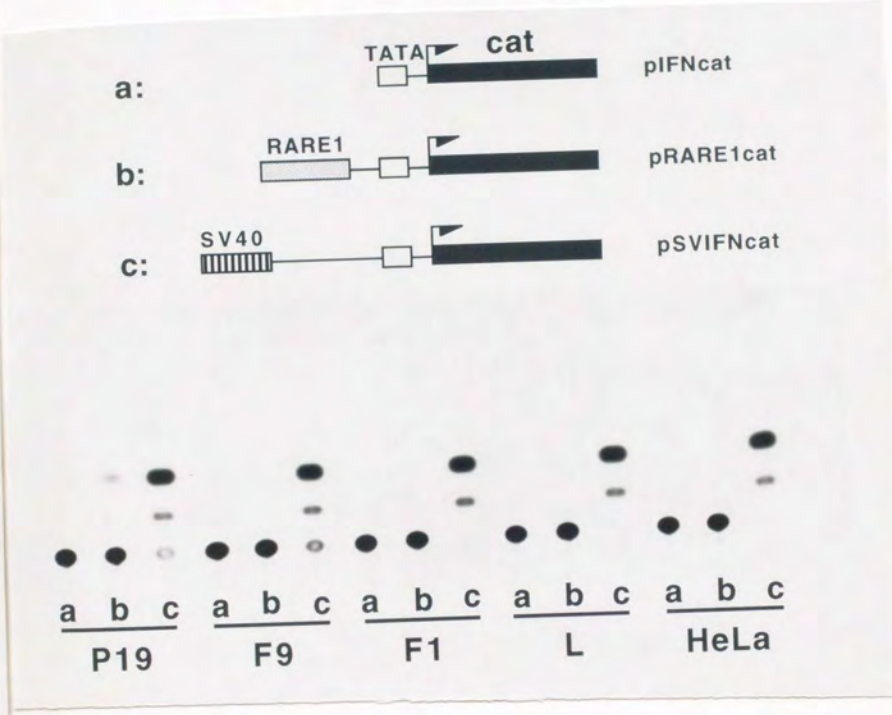

a:
TATA
cat
pIFNcat
b:
RARE1
pRARE1cat
c:
SV40
pSVIFNcat
a b c
P19
a b c
F9
a b c
F1
a b c
L
a b c
HeLa

Figure II-2. *RARE1 blocks the activity of SV40 enhancer in cell-type-specific manner.* The illustrated constructs were transfected into indicated cell lines and the resulting CAT activities obtained are shown. a: described in the legend to Figure III-1. b: the RARE1 fragment was inserted between the SV40 enhancer and the TATA box. RA-P19 : P19 cells were cultured in the presence of 1 μM RA from the transfection untill cell harvest.

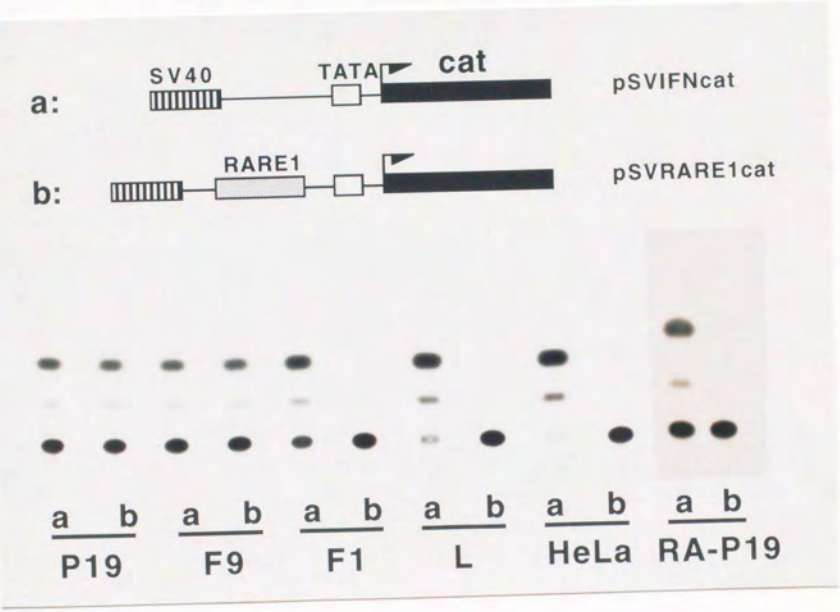

a:
SV40
TATA
cat
pSVIFNcat
b:
RARE1
pSVRARE1cat
a b
P19
a b
F9
a b
F1
a b
L
a b
HeLa
a b
RA-P19

Figure II-3. *Localization of negatve regulatory elements in RARE1 region.* **(A)** The sequence of the RARE1 region (-1132 to -890) previously published (Okazawa et al., 1991). The name of each deletion construct is indicated above the sequence, with a right- or a left-angled arrow to specify the precise endpoint of the construct. The regions containing the negative regulatory activity (SE-A: -1081 to -1014 and SE-B: -936 to -894) are shown in bold print. **(B)** The illustrated deletion constructs were tested by transfection into HeLa cells and CAT assays. The histogram show fold-repression calculated as the ratio between CAT activity from each deletion construct and that from pSVIFNcat.

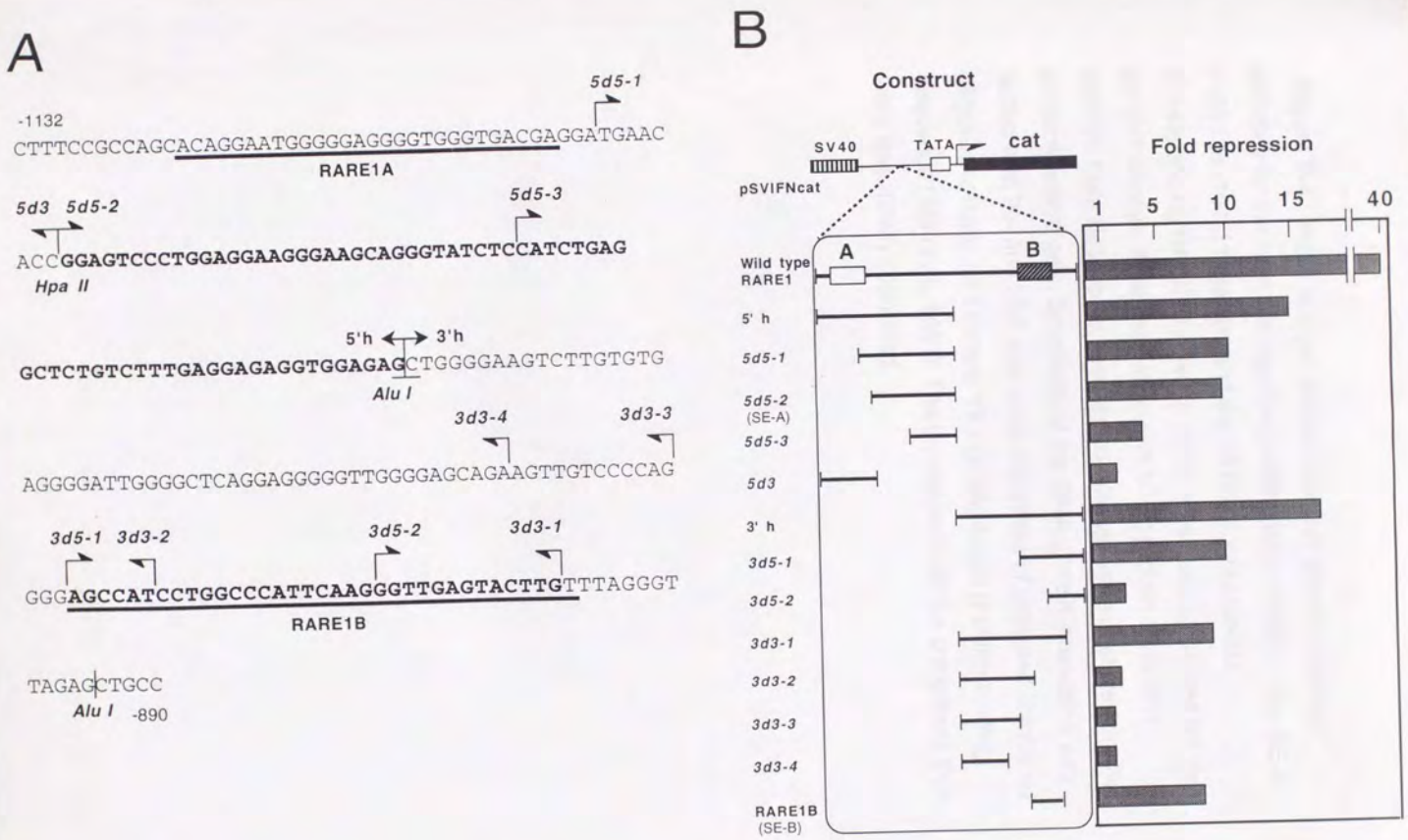

A
5d5-1
-1132
CTTTCCGCCAGCACAGGAATGGGGGAGGGGTGGGTGACGAGGATGAAC
RARE1A
5d3
5d5-2
5d5-3
ACCGGAGTCCCTGGAGGAAGGGAAGCAGGGTATCTCCATCTGAG
Hpa II
5'h
3'h
GCTCTGTCTTTGAGGAGAGGTGGAGAGCTGGGGAAGTCTTGTGTG
Alu I
3d3-4
3d3-3
AGGGGATTGGGGCTCAGGAGGGGGTTGGGGAGCAGAAGTTGTCCCCAG
3d5-1
3d3-2
3d5-2
3d3-1
GGGAGCCATCCTGGCCCATTCAAGGGTTGAGTACTTGTTTAGGGT
RARE1B
TAGAGCTGCC
Alu I
-890
B
Construct
SV40
TATA
cat
pSVIFNcat
Fold repression
1
5
10
15
40
Wild type RARE1
A
B
5' h
5d5-1
5d5-2 (SE-A)
5d5-3
5d3
3' h
3d5-1
3d5-2
3d3-1
3d3-2
3d3-3
3d3-4
RARE1B (SE-B)

Figure II-4. *HeLa nuclear extracts contain specific binding activities for the negative regulatory elements in RARE1.* The SE-A (-1081 to -1014) fragment and the 35 bp oligonucleotide corresponding RARE1B (-943 to -909) were used as probes for the gel shift assays. After incubation with 10 μg protein of nuclear extracts from HeLa cells, resulted complexes were analyzed on 4% polyacrylamide gels. Specificity of the DNA-protein interaction was tested with 20- and 200- fold molar excesses of indicated unlabeled oligonucleotides. YY1 means YY1 (a zinc-finger protein) binding sequence (Shi et al., 1991). The arrows indicate the complexes that were specifically competed.

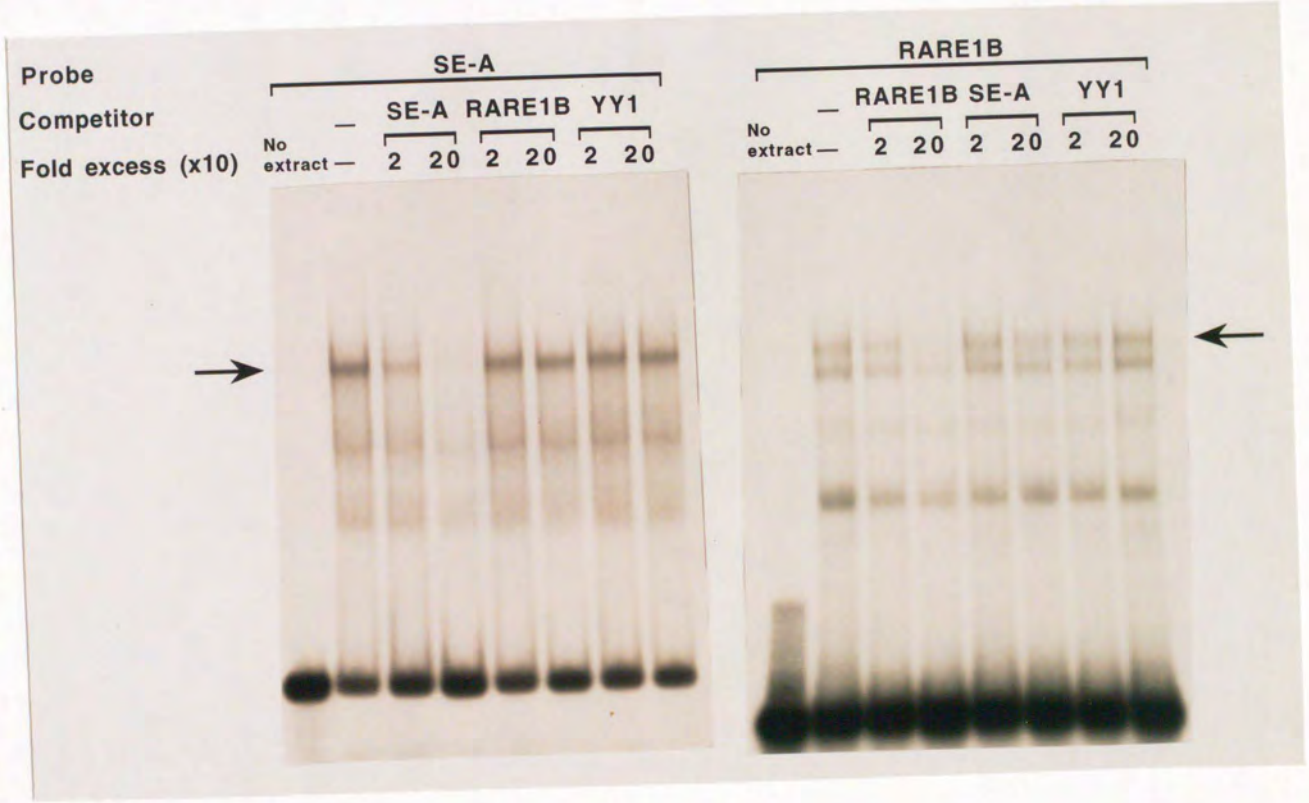

Probe
SE-A
RARE1B
Competitor
—
SE-A
RARE1B
YY1
No extract
—
2
20
2
20
2
20
No extract
—
RARE1B
SE-A
YY1
—
2
20
2
20
2
20

Figure II-5. *Analysis of SE-A element.* **(A)** DMS - interference assay of the SE-A binding protein. Partially methylated SE-A containing DNA fragments labeled on coding or noncoding strand were used in gel shift assay with nuclear extracts from HeLa cells. The bound and free DNA fragments were eluted from the gel, treated with piperidine, and fractioned on 10% sequencing gel. Two effective regions for the complex formation are indicated (1 and 2) and the sequences are shown. The closed and open circle (left side of the sequences) indicate the G residues whose methylation strongly or weakly interferes, respectively, with the formation of the complex. **(B)** Competition analysis in gel shift assay of SE-A binding proteins in nuclear extracts from HeLa cells. The 27 bp oligonucleotide containing SE-A1 (-1086 to -1061) and the 27 bp oligonucleotide containing SE-A2 (-1035 to -1009) were used as probes. Indicated unlabeled oligonucleotides were used as competitors with 20- and 200- fold molar excesses. Sp1 means the recognition sequences for a transcription factor Sp1 (Kadonaga et al., 1987). The arrows indicate specific retard complexes.**(C)** Functional analysis of SE-A. The illustrated conctructs were tested by transfection into HeLa cells and CAT assays. The oligonucleotides containing SE-A 1and 2 described above were inserted between the SV40 enhancer and the TATA box of pSVIFNcat in either orientation, respectively. The calculation of fold repression was described in the legend to Figure III-3.

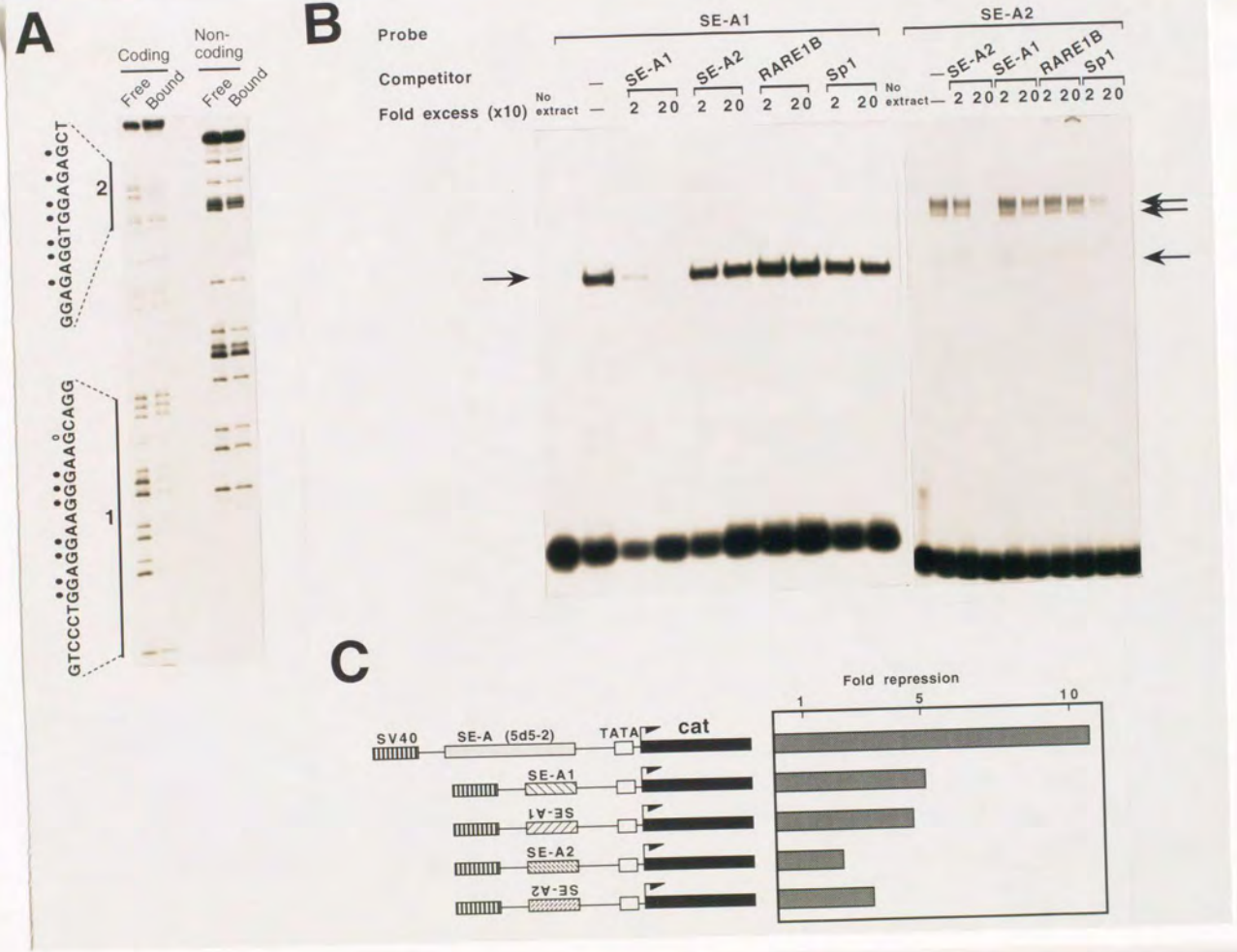
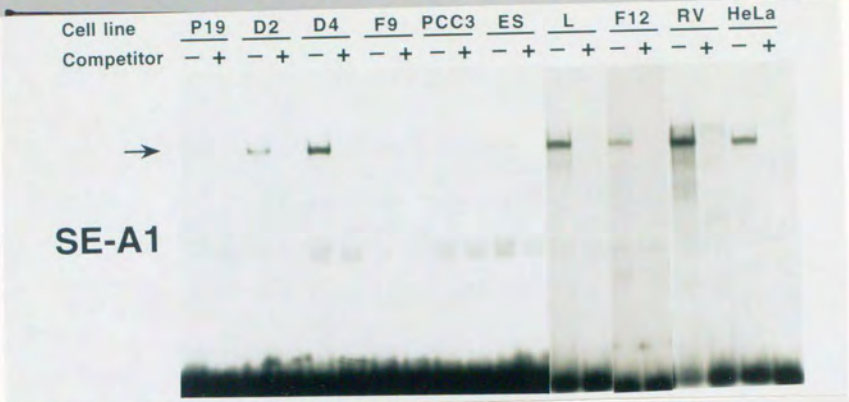
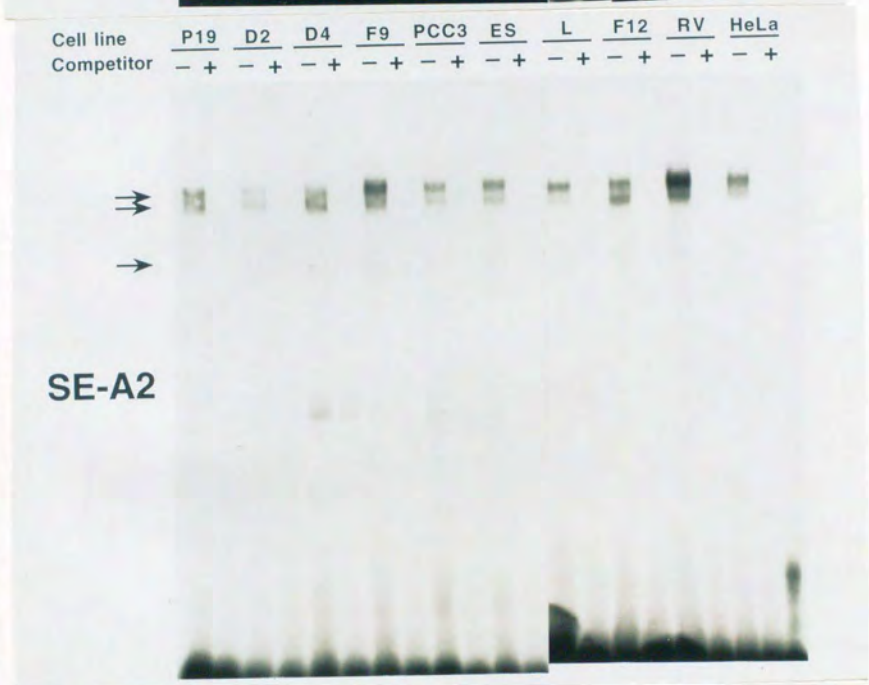

A
Coding
Non-coding
Free
Bound
Free
Bound
GGAGAGGTGGAGAGCT
2
GTCCCTGGAGGAAGGGAAGCAGG
1
B
Probe
SE-A1
SE-A2
Competitor
—
SE-A1
SE-A2
RARE1B
Sp1
No extract
Fold excess (x10)
2 20 2 20 2 20 2 20
C
SV40
SE-A (5d5-2)
TATA
cat
SE-A1
SE-A2
Fold repression
1
5
10

Figure II-6. *Gei shift analysis of binding activities for the negative regulatory elements in RARE1with nuclear extracts from various cell lines.* The indicated oligonucleotide probes were incubated with each nuclear extracts (8 μg of protein) from the various cell lines and analyzed by gel shift assay. The specificity of the complex formations were confirmed with competition by 200- fold molar excess of unlabeled oligonucleotides corresponding to the probes. The arrows indicate specific retard complexes.


Cell line
P19 D2 D4 F9 PCC3 ES L F12 RV HeLa
Competitor
− + − + − + − + − + − + − + − + − + − +
SE-A1


Cell line
P19 D2 D4 F9 PCC3 ES L F12 RV HeLa
Competitor
− + − + − + − + − + − + − + − + − + − +
SE-A2

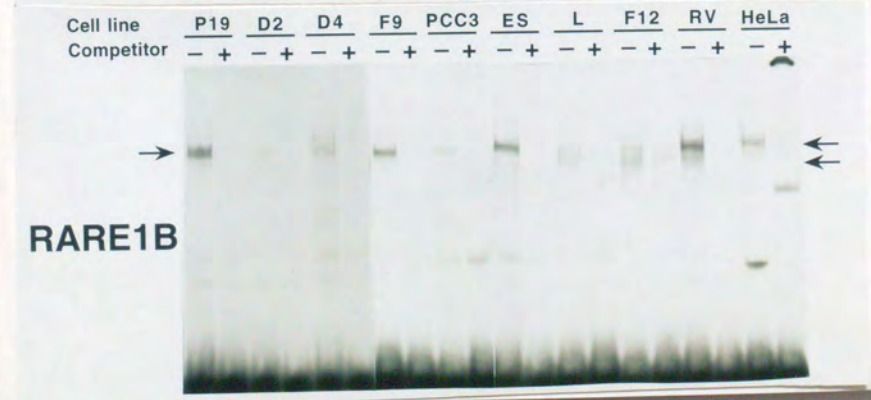
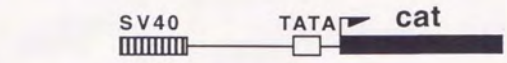
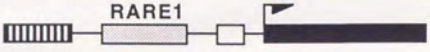
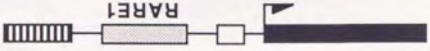

Cell line
P19 D2 D4 F9 PCC3 ES L F12 RV HeLa
Competitor
− + − + − + − + − + − + − + − + − + − +
RARE1B

Table I-1. *Summary of various cell lines and their phenotype*

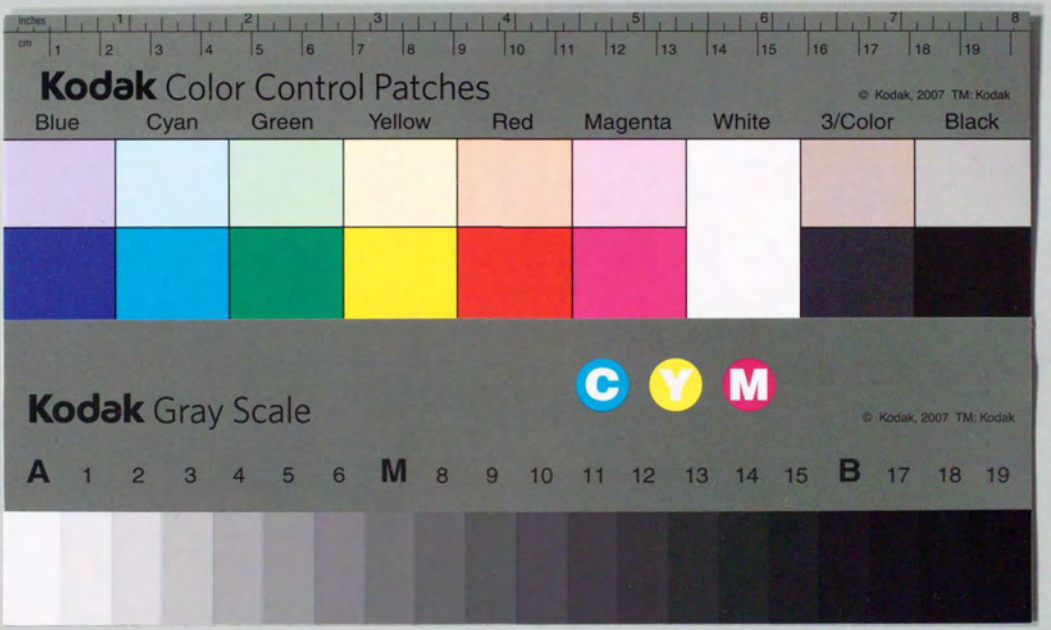
Cell line	G418 resistance	Oct-3 expression	Differentiation markers		Potetial to differentiate
			SSEA-1	nestin & Brn-2	
P19 (052 HGPRT)	+	+	+	−	+
L-TK	n/a	−	−	−	−
Hybrid (F1)	−	−	−	+	−
Revertants (RV-1 and 2)	+	+	−	−	+
Defferentiated revertants (dRV-1 and 2)	−	−	−	+	−
Re-revertants (rRV-1 and 2)	+	+	−	−	+
Differentiated re-revertants (drRV-1 and 2)	−	−	−	+	−
F1-E1a	n/a	−	−	+	−

Phenotypes of various cell lines used in this study are summarized. n/a: applicable. See text for details

Table II-1. *The ability of RARE1 to block the activity of the SV40 enhancer depends on relative position of the enhancer, promoter, and RARE1 site*

Reporter construct	Relative CAT activity
	100.0
	1.3
	13.6
	66.4
	19.7
	2.2

The relative CAT activity resulting from tansfection of reporter genes into HeLa cells are listed. The reporters diagramed, indicating the relative positions of the SV40 enhancer, TATA box from the IFN β promoter (-55 to +19), and RARE1 site. RARE1 means reverse orientation form of the RARE1.


inches
cm
Kodak Color Control Patches
© Kodak, 2007 TM: Kodak
Blue
Cyan
Green
Yellow
Red
Magenta
White
3/Color
Black
Kodak Gray Scale
C
Y
M
© Kodak, 2007 TM: Kodak
A 1 2 3 4 5 6 M 8 9 10 11 12 13 14 15 B 17 18 19