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

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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 ('revertants'). 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.

Key words: embryogenesis/hybrid cell extinction/Oct-3/POU

Introduction

We have been interested in identifying regulatory genes involved in mammalian early embryogenesis, and have recently isolated one such candidate gene, *Oct-3* (Okamoto *et al.*, 1990). The same gene (also called *Oct-4*) was isolated by other groups using a different approach (Rosner *et al.*, 1990; Schöler *et al.*, 1990b). The gene encodes an octamer-binding transcription factor, which has a class V POU domain (Herr *et al.*, 1988) as a DNA-binding domain, and a transactivation domain in the amino-terminal region (Okamoto *et al.*, 1990; Imagawa *et al.*, 1991). The most interesting feature of this gene is its expression pattern; it is expressed exclusively in totipotent/pluripotent early embryonic cells (such as inner cell mass cells) and some germ cells (Rosner *et al.*, 1990; Schöler *et al.*, 1990b). The *Oct-3* gene 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; Rosner *et al.*, 1990; Schöler *et al.*, 1990b). Although there is no direct evidence that the *Oct-3* gene plays a role in early embryogenesis, its expression pattern suggests that it may be required for maintaining pluripotency and that the down-regulation of the *Oct-3* gene may be required for subsequent differentiation.

We have been using the P19 EC cell line as a model system

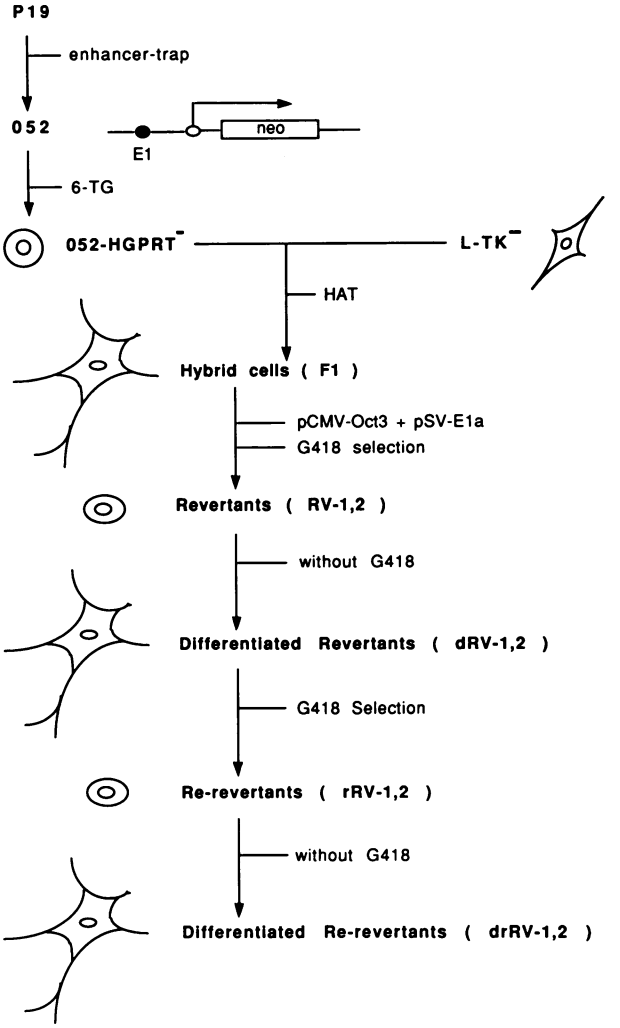

Fig. 1. Experimental strategy for selecting the hybrid cells and their derivative cell lines. The strategy for selecting the hybrid cell lines and their derivatives is summarized. Cell line 052 is transformed with one copy of an enhancer-trap, an enhancerless plasmid with the *neo* gene (Bhat *et al.*, 1988). The trap is integrated near the endogenous transposon called 'early transposon' (Brulet *et al.*, 1983). The *neo* 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 detail.

for studying 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; Okazawa *et al.*, 1991). 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, and more importantly, the Oct-3 protein alone is unable to activate an octamer-enhancer, due to its cell-type specific activation domain (as described in Results), indicating that the Oct-3 protein needs another factor to exert its function. This means that experiments involving ectopic expression of *Oct-3* alone would not be sufficient to see the effects of Oct-3 function.

In order to overcome these problems, we have devised a system which can select cells depending on the presence or absence of Oct-3 transactivating function. First, P19 cells which had been transformed with an enhancer-trap were fused to L cells, and hybrid cell lines were established. The hybrid cells were found to have differentiated into neuroepithelial-like cells, and accordingly the *Oct-3* gene was extinguished. We 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 1. Generally, when two different cell types are fused, expression of many cell-type specific genes is repressed in the hybrid cells (Davidson, 1974; Brown and Weiss, 1975). This phenomenon is known as hybrid cell extinction. We 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 a certain differentiated phenotype. From the hybrid cells, we 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 which 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 differentiate 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 2). The

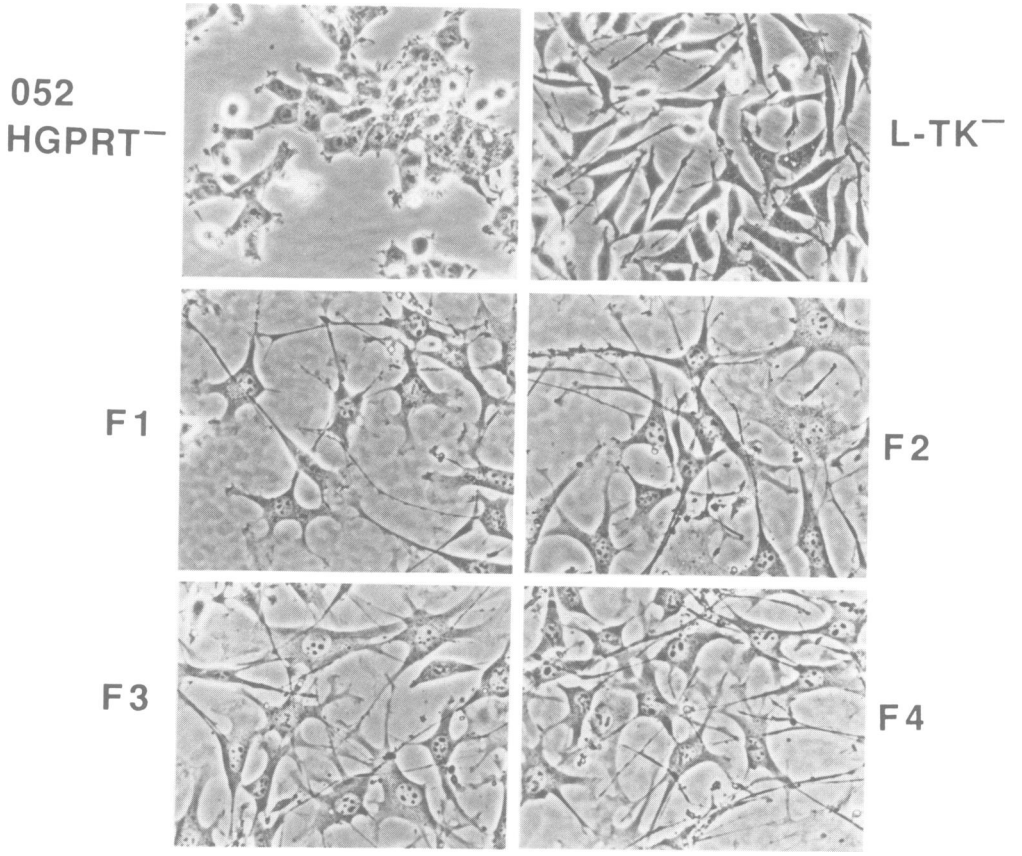

Fig. 2. Morphology of parental cell lines and hybrid cell lines. Phase contrast photographs of two parental cell lines (052HGPRT$^-$ and L-TK$^-$) and four hybrid cell lines (F1, F2, F3 and F4) are shown.

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 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 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 4), while it did not stain the cytoskeleton of P19 or L cells (data not shown).

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 (H.Fujii and H.Hamada, submitted). Gel shift analysis of nuclear extracts from the hybrid cells detected Oct-1 and another binding factor (Figure 5A, lanes F1, F2 and F3). The latter octamer-binding factor is most likely to be Brn-2, for the following reasons. First, this binding factor comigrated with the authentic protein which was produced from the cloned Brn-2 expression vector in HeLa cells (data not shown). Secondly, *Brn-2* mRNA was detected in the hybrid cells by a semi-quantitative reverse PCR assay (data not shown). Finally, an antibody against Brn-2 could recognize and super-shift the binding factor (Figure 5B).

We 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.

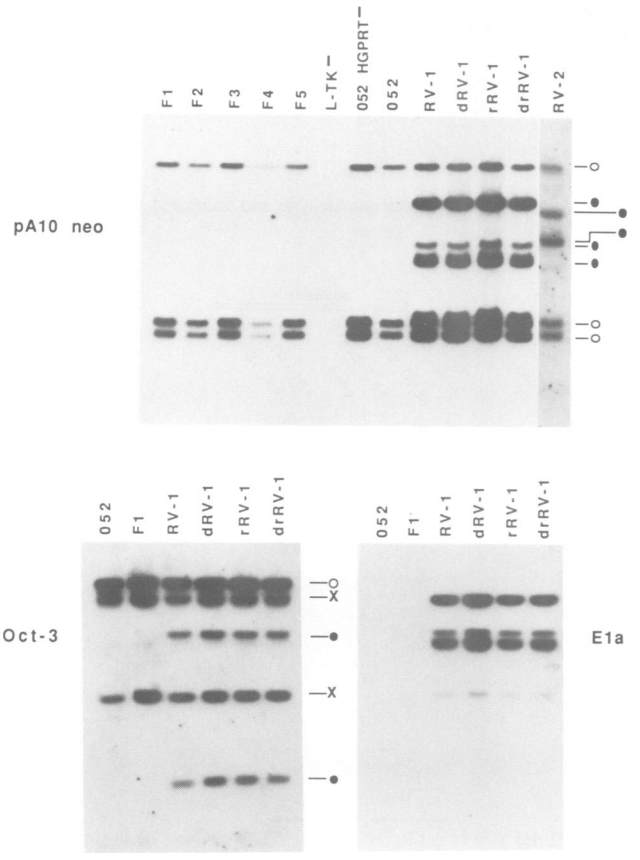

Fig. 3. The enhancer-trap copy is stably maintained during the cell-fusion and G418-selection. Genomic DNA from the indicated cells was digested with *Eco*RI and subjected to Southern blot. Three probes were used; the enhancer-trap plasmid (pA_{10}neo; Bhat *et al.*, 1988), *Oct-3* and *E1a*. Since the 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 the 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.

Oct-3 expression is extinguished in the hybrid cells

We 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 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 (data not shown). Therefore, the antibody appears to be specific to Oct-3 protein. When the level of Oct-3 in the hybrid cells was examined with the antibody, Oct-3 protein was undetectable in any of the 20 hybrid cell lines (Figure 6B and data not shown).

The level of Oct-3 protein was also determined by gel-shift assay, and it was again undetectable in the hybrid cell lines (Figure 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 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 the *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 close to the TATA box. In this study, we have examined whether Oct-3 can stimulate the enhancer-dependent transcription from a distal binding site. When the octamers (26 bp sequences derived from E1) were placed 0.1 kb away from the SV40 promoter, these sequences acted as a cell-type specific enhancer; pOCTAcat, relative to pBScat, was transcriptionally active in P19 cells (Figure 8, lanes 1 and 2), but inactive in HeLa and F1 cells (Figure 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 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

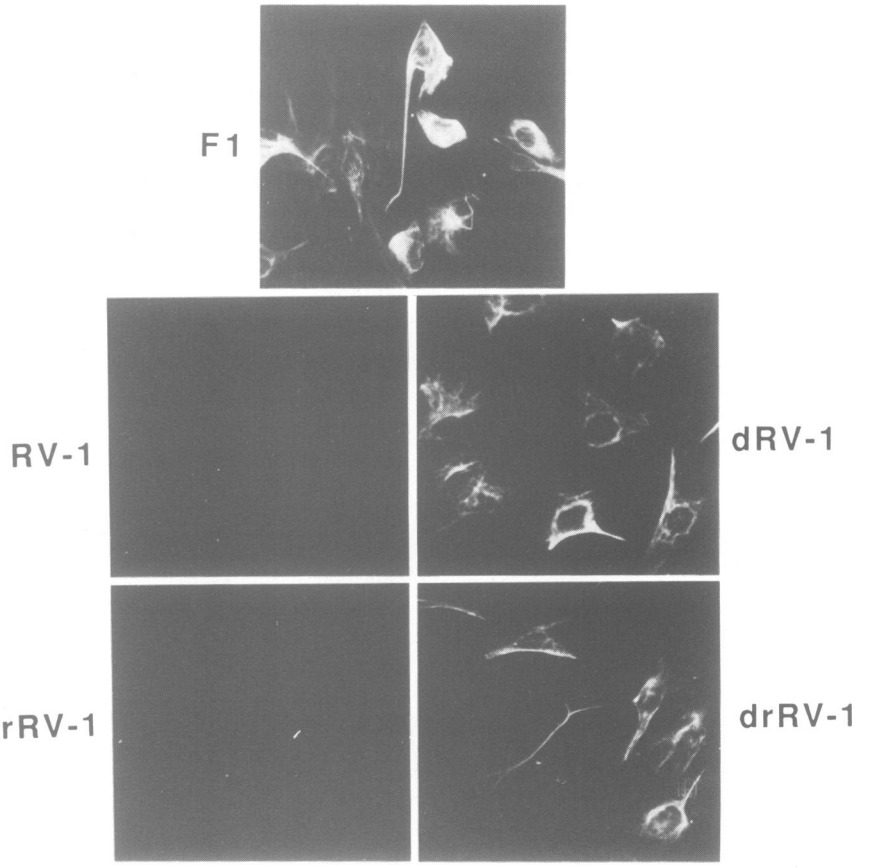

Fig. 4. Inverse correlation between Oct-3 and nestin expression. The indicated cells were fixed with paraformaldehyde and incubated with an anti-nestin antibody. Nestin was visualized using an FITC-conjugated second antibody.

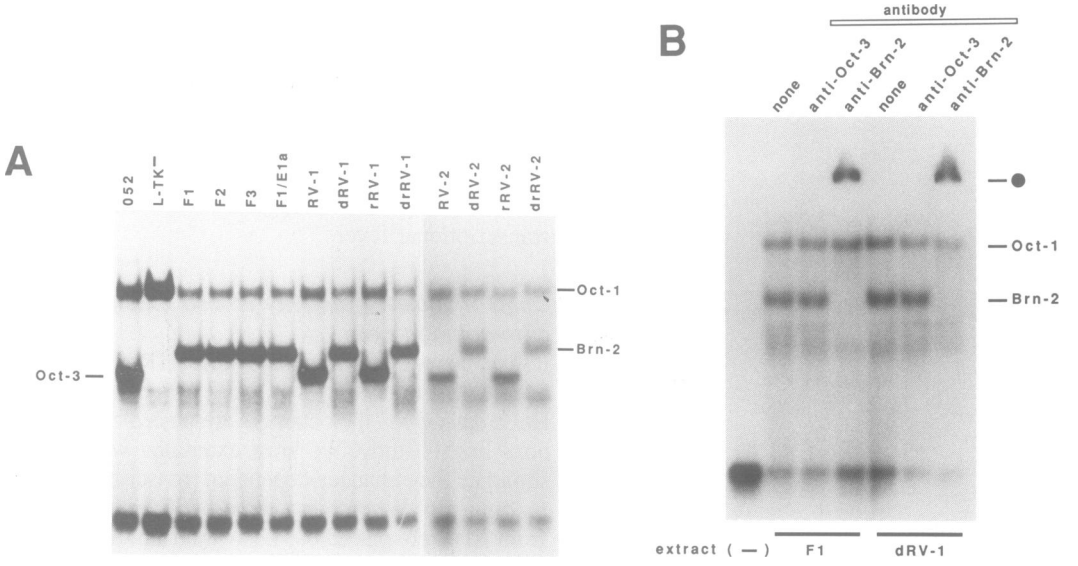

Fig. 5. Inverse correlation between Oct-3 and Brn-2 expression. (**A**) Nuclear extracts were prepared from the indicated cells. The octamer-binding activity in each extract was examined by gel-shift assay. Three octamer-binding factors (Oct-1, Oct-3 and Brn-2) are indicated. Note that the expression 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 circles.

regulates the immunoglobulin enhancer in EC cells. Western blot analysis confirmed that a considerable amount of Oct-3 protein was produced in the transfected cells (Figure 8, lanes 8 and 9). Furthermore, gel shift assay showed that the Oct-3 protein produced in HeLa cells was capable of binding to the octamer sequence (data not shown). 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. In order to confirm this, the Oct-3 transactivating domain, the amino-terminal region of this

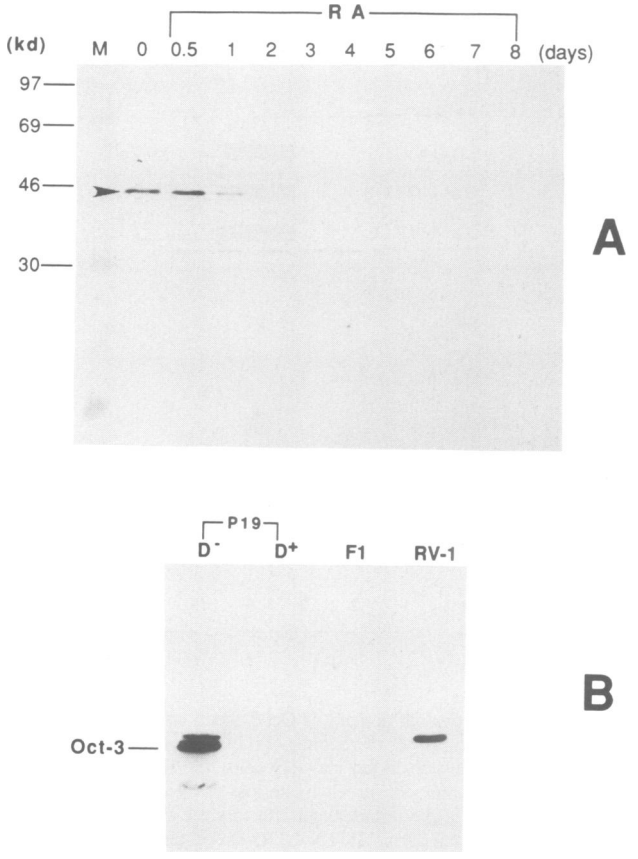

Fig. 6. The level of Oct-3 protein in various cells. (**A**) P19 cells were induced to differentiate with RA. Cell lysates were prepared at the indicated time after the induction, and Oct-3 protein level was assayed by Western blot. The 43 kDa protein (Oct-3) is indicated by the arrow. Note that the 43 kDa protein was detected in P19 cells but disappeared 24 h after the induction. (**B**) Cell lysates were also prepared from untreated 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.

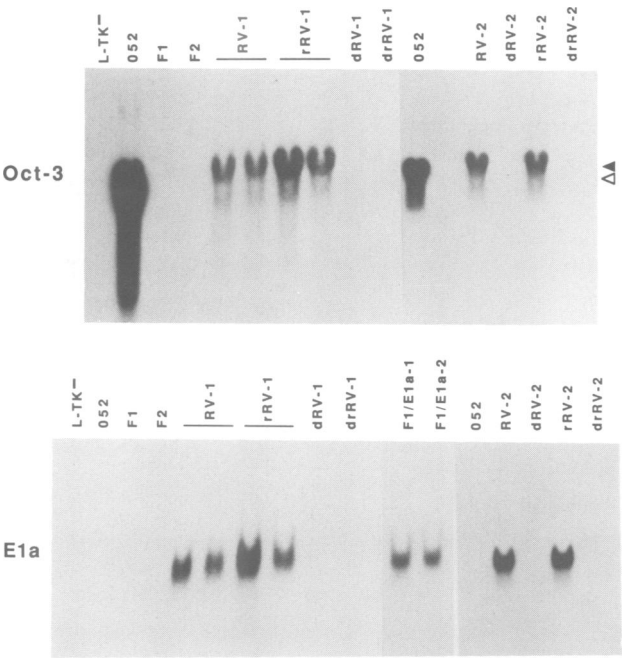

Fig. 7. Northern blot analysis of *Oct-3* and *E1a* mRNA. 20 μg of cytoplasmic RNA from various cell lines were subjected to Northern blot analysis. For RV-1 and rRV-1, two different batches of RNA were examined. Note that *Oct-3* mRNA expressed in the RV-1, rRV-1, RV-2 and rRV-2 cells is apparently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

protein (Okamoto *et al.*, 1990; Imagawa *et al.*, 1992), was fused to the DNA-binding domain of GAL4. Such a chimeric protein (GAL4/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 9). GAL4/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$^-$ cells 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 (Figures 5A, 6B and 7). Therefore, the endogenous *neo* gene was inactive and the hybrid cell lines were sensitive to G418 (Table I). If some of the hybrid cells regain Oct-3 function, such cells ('revertants') should become resistant to G418. Initially, we 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 2×10^7 cells. Subsequently, *Oct-3* was ectopically introduced into the hybrid cells. When the F1 cells were transfected with the Oct-3 expression vector (pCMVOct3) alone and then cultured in the presence of G418, no G418-resistant cells were rescued (less than 1 in 2×10^7 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 (Figures 8 and 9). It should be noted that the E1 enhancer is located 1 kb upstream of the *neo* gene promoter. Recently, 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. Based on their observations, we 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 4×10^7 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 3, and data not shown). 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 6B and data not shown) and by gel-shift assay (Figure 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 7). The *Oct-3* mRNA detected in the RV-1 and RV-2 cells was larger than the transcript in P19 cells (Figure 7),

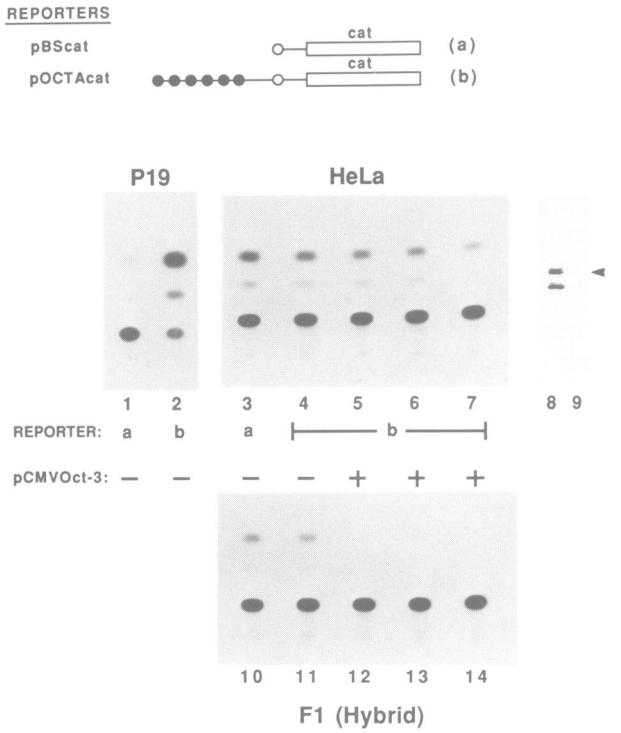

Fig. 8. Oct-3 alone cannot stimulate the enhancer-dependent transcription in differentiated cells. Structures of two reporter CAT plasmids are shown on top. pOCTAcat (a) contains six tandem copies of octamer-motif sequences (closed circles) linked to the enhancerless SV40 early promoter (open circle). The other reporter, pBScat (b), lacks the octamer-motif sequences. In lanes 1−7 and 10−14, one of the reporter plasmids was transfected into the 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 Materials and methods. In lanes 8 and 9, Oct-3 protein produced in HeLa cells was analysed 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.

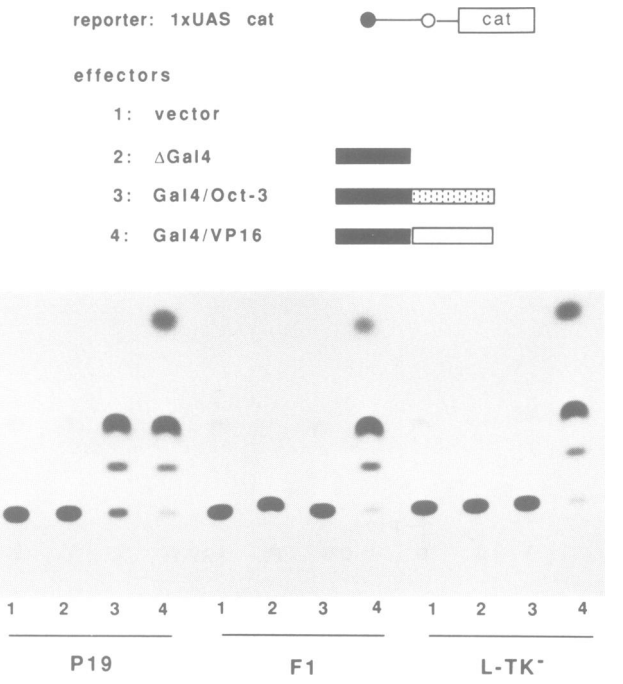

Fig. 9. The transactivating domain of Oct-3 functions in a strictly cell-type specific fashion. 10 μg of reporter (1xUAScat) and 5 μg of one of the effectors were transfected into P19 cells, the F1 cells or L-TK$^-$ cells. An equivalent amount of cell lysate was assayed for CAT activity. Structures of 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.

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 6B) and Northern blot (Figure 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, we 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 a round cell body and did not have long processes (Figure 10). While the F1 cells grew dispersely, the RV-1 cells were more adherent (Figure 10). The RV-1 cells appeared rather similar to P19 cells in morphology. Furthermore, the RV-1 cells were negative for nestin (Figure 4) and Brn-2 (Figure 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 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 similar to that of the F1 cells; the dRV-1 cells possessed multiple long processes (Figure 10), and were positive for nestin (Figure 4) and Brn-2 (Figure 5A and B).

Since the dRV-1 cells lacked *Oct-3* function, they were sensitive to 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 2×10^3 cells. 5×10^2 G418-resistant colonies were pooled and they were designated as rRV-1 ('re-revertant'). The phenotype of the rRV-1 cells was indistinguishable from that of RV-1. The rRV-1 cells were morphologically similar to the RV-1 cells (Figure 10). While the rRV-1 cells had regained the expression of the *Oct-3* and *E1a* genes (Figure 7), they had lost the expression of Brn-2 (Figure 5A) and nestin (Figure 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 7), and were positive for Brn-2 (Figure 5A) and nestin (Figure 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 the RV-1 cells (Figure 10) and were Oct-3$^+$ (Figure 5A), Brn-2$^-$ (Figure 5A) and nestin$^-$ (data not shown). Furthermore, when the RV-2 cells were transferred to medium lacking G418, they lost expression of the *Oct-3* and *E1a* genes.

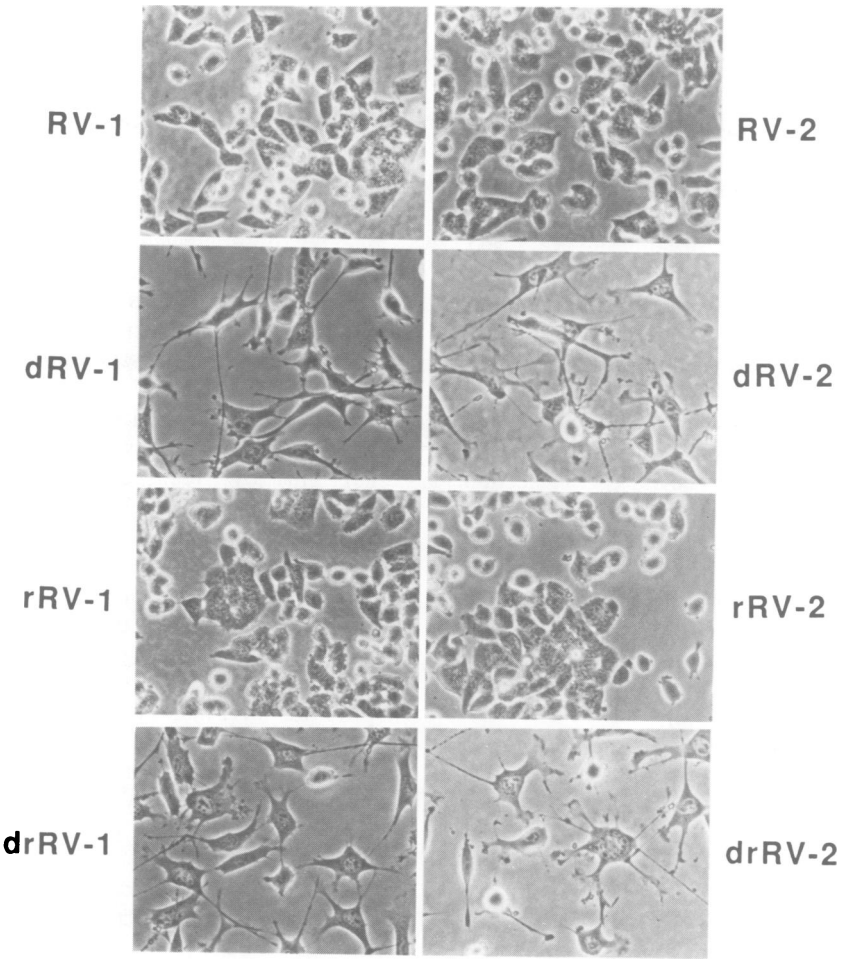

Fig. 10. Morphology of two 'revertant' cell lines and their derivative cell lines. Phase contrast photographs of two 'revertant' cell lines (RV-1 and RV-2) and their derivative cell lines are shown. See text for details.

Table I. Summary of various cell lines and their phenotype

	G418 resistance	Oct-3 expression	Differentiation markers		Potential to differentiate
			SSEA-1	nestin and Brn-2	
P19 (052 HGPRT$^-$)	+	+	+	−	+
L-TK$^-$	n/a	−	−	−	−
Hybrid (F1)	−	−	−	+	−
Revertants (RV-1 and -2)	+	+	−	−	+
Differentiated 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: not applicable. See text for details.

Accordingly, these cells transformed to Oct-3$^-$/nestin$^+$/Brn-2$^+$ cells (dRV-2), which were G418-sensitive. When the dRV-2 cells were returned to the G418-containing medium, G418-resistant cells (rRV-2) appeared at a frequency of 1 in 5×10^2 cells. The rRV-2 cells were Oct-3$^+$/Brn-2$^-$/nestin$^-$ (Figures 5 and 7, and data not shown). When the rRV-2 cells were maintained in the absence of G418, the majority of the cells transformed backed to Oct-3$^-$/Brn-2$^+$/nestin$^+$ cells (drRV-2). 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 G418-lacking medium 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 7), we were concerned with the possibility that the phenotypic changes might be simply due to *E1a* expression. In order 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 transformants expressing *E1a* mRNA were obtained. They were all morphologically indistinguishable from the F1 cells (data not shown). Two such cell lines (F1/E1a-1 and -2) expressing *E1a* mRNA at a comparable level to the RV-1 cells (Figure 7) were further examined, and both were nestin$^+$/Brn-2$^+$ (Figure 5 and data not shown). 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. We have also examined SSEA-1, a marker specific to EC stem 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 our G418-selection described here was based on the transactivating function of Oct-3, we 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 gene extinction

P19 cells can respond to chemical reagents such as RA and DMSO, and differentiate into various cell types (Jones-Velleneuve *et al.*, 1983). *Oct-3* is switched off at an early stage of the differentiation process. The present study has shown that the fusion to fibroblasts can also initiate differentiation and *Oct-3* extinction [after this manuscript was submitted, Ben-Shushan *et al.* (1993) reported Oct-3 extinction in cell hybrids between F9 EC cells and fibroblasts]. The simplest explanation for this would be that a 'differentiation-inducing' gene is active in fibroblasts and that this can act dominantly upon the cell fusion. If this is the case, it may be possible to reproduce similar differentiation by introducing a single chromosome (instead of a whole set of chromsomes) from fibroblasts into P19 cells.

The expression of the *Oct-3* gene is regulated by a stem cell-specific/RA-repressible enhancer (RARE1) located 1 kb upstream of the gene (Okazawa *et al.*, 1991); RARE1 is required for active expression in undifferentiated P19 cells and confers the RA-mediated repression. Recent findings of ours (Shimazaki *et al.*, unpublished) suggested that the hybrid cell extinction of the *Oct-3* gene also involves RARE1. Therefore, RARE1 may be regarded as a stem cell-specific and differentiation-sensitive enhancer. Furthermore, differentiated cells possess a unique RARE1-binding factor, RABPβ (Shimazaki *et al.*, unpublished). Since the presence of RABPβ was inversely correlated with *Oct-3* expression in every case examined, this binding protein may act as a strong repressor of the *Oct-3* gene. It is possible that when P19 cells were fused to L cells, RABPβ present in L cells repressed the transcriptionally active *Oct-3* gene in the P19 cells.

We 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 (Figures 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 activating domain. One is that the Oct-3 transactivating domain needs to interact with an adaptor, which is present only in ES cells. The other possibility is that the Oct-3 transactivating domain itself is active but differentiated cells possess 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 Oct-3 transactivation 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 adaptor for Oct-3, E1A induced the adaptor, or E1A abolished the interaction with an Oct-3 masking factor.

Role of Oct-3 in embryonic stem cell differentiation

In our strategy for selecting cell lines, G418-resistance/sensitivity directly reflects the presence or absence of the Oct-3 function. Therefore, any phenotypic differences between the hybrid cells and the revertants (and also 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 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 in order 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 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). This is in good agreement with the behaviour 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 *et al.*,

unpublished). However, it is not certain whether these two genes are under the direct control of *Oct-3*. We have recently found that there is at least one binding site for Oct-3 in the upstream region of the *Brn-2* gene (our unpublished data), but it remains to be seen whether this binding site acts as a negative *cis* element for *Brn-2* expression.

The RV-1 and RV-2 cells resembled P19 cells rather than the F1 cells (Figure 10). However, there was a difference between the RV cells and P19 cells: P19 cells were positive for SSEA-1 whereas RV cells were negative (Table I). Therefore, the RV cells are not complete revertants of the F1 cells (we tried to examine other EC cell-specific markers, but unfortunately such markers are limited). Obviously, some of the phenotype of P19 EC cells was 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) 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-1 cells. The cell lines obtained in this study (such as F1, RV-1 and dRV-1) may be useful for selecting Oct-3-regulated genes.

In summary, we have shown that P19-L cell hybrids undergo differentiation into neural cells in parallel with the extinction of the *Oct-3* gene. Furthermore, the ectopic introduction of Oct-3 function into the 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.

Materials and methods

Materials

The Oct-3 expression vector (pCMVOct-3) was constructed by inserting the full-length *Oct-3* cDNA (Okamoto *et al.*, 1990) into 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 Institute of Medical Sciences, University of Tokyo).

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 us (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 (200 μg/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 200 μg/ml of G418. One G418-resistant colony (RV-1) appeared on a dish that received 10 μg of pCMVOct-3 and 5 μg 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 medium lacking G418 and were maintained in the absence of G418 for 7 days. A whole cell population that was exposed for the non-selective condition for 7 days 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 200 μg/ml of G418. Although most of the dRV-1 cells were killed within 7 days, G418-resistant colonies appeared at a frequency of 1 in 2×10^3. About 5×10^2 G418-resistant colonies were pooled and maintained in the presence of G418 (designated as rRV-1). The drRV-1 cells were isolated from rRV-1, 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 to dRV-2 and drRV-2, respectively. Unless otherwise mentioned, all the hybrid cell lines, dRV-1, drRV-1, dRV-2 and drRV-2, were maintained 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 expressing 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-1 and -2), expressing *E1a* mRNA at a comparable level to the RV-1 cells, were used as controls in this study.

CAT assays

The GAL4/Oct-3 expression vector was constructed as described below. The Oct-3 coding region (codons 1−134) was obtained by PCR as an *Xba*I−*Bam*HI 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 activating domain is fused to the carboxyl-terminus of the GAL4 DNA binding domain (codons 1−147). GAL4/VP16 expression vector (Sadowski *et al.*, 1988) was kindly provided by Dr M.Ptashne. A reporter plasmid containing a GAL4-binding site (1xUAS-cat; Yoshimura *et al.*, 1989) was kindly provided by Drs J.Fujisawa and M.Yoshida. Transfection was performed essentially as described by Chen and Okayama (1984). In most of the transfection experiments, pCH110 (a *lacZ* reporter driven by the SV40 enhancer/promoter) was included as an internal standard in order to normalize the transfection efficiency. CAT assay was performed as described by others (Gorman *et al.*, 1982).

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 glutathione−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 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 an antigen (Lendhal *et al.*, 1990). For histochemical staining of nestin, cells were fixed with 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.

DNA-binding assays

Nuclear extracts were prepared essentially as described by Schreiber *et al.* (1990). An oligonucleotide containing an octamer motif (26mer; GATCAGTACTAATTAGCATTATAAAG) was end-labelled and used as a probe. Gel-shift assays were performed as described previously (Okamoto *et al.*, 1990). For the supershift assay, the anti-Brn-2 antibody was raised in a rabbit with the GST/Brn-2 fusion protein as an antigen (H.Fujii and H.Hamada, submitted). The antibody was purified by passing it through a GST−Sepharose column followed by binding to protein A−Sepharose.

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