Octamer binding proteins confer transcriptional activity in early mouse embryogenesis

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Oct4 and Oct5 are two mouse maternally expressed
proteins binding to the octamer motif. Both are found
in unfertilized oocytes and embryonic stem cells, whereas
Oct4 is also found in primordial germ cells. In this study,
the activity of the octamer motif was analysed in two
embryonic stem cell lines containing Oct4 and Oct5, the
teratocarcinoma-derived cell line F9 and the blastocyst-
derived cell line D3. It is known that oligomerization of
the octamer motif creates a powerful B-cell specific
enhancer. As shown here, this oligomerized transcrip-
tional element is also a very strong enhancer in F9 and
D3 embryonic stem cells. After differentiation of the stem
cells, both enhancer activity and the amount of the
octamer binding proteins decrease. An intact octamer
stimulates heterologous promoters in embryonic stem
cells, whereas mutations in the octamer motif abolish
transcriptional stimulation and binding of the octamer
factors. The use of transgenic embryos demonstrates
transcriptional activation in the inner cell mass but not
in the trophoblast of blastocysts. The results indicate that
Oct4 and Oct5 are active early in mouse development.
Key words: embryogenesis/enhancer/maternal/octamer/
transcription factor

Introduction

The molecular mechanisms that regulate mammalian
embryogenesis are poorly understood. It is assumed that a
network of regulatory genes controls the transformation of
genetic information into embryonic structure. During recent
years a number of mouse homeobox and paired-box
containing genes were identified on the basis of sequence
similarity to *Drosophila* genes (for review see Dressler and
Gruss, 1988). While the expression patterns (for refs see
Holland and Hogan, 1988) and mutational transgene studies
concerning Pax and Hox genes (Balling *et al.*, 1988, 1989)
indicate a role in pattern formation, developmental activa-
tion of these genes is restricted to post-implantation stages
of mouse development. Moreover, the exact function and
target sites remain unknown (Dressler and Gruss, 1988).

Oct1 and Oct2 are mammalian homeo-domain containing
proteins whose target sequence is known, and are regarded
to be ubiquitous and cell-specific transcription factors
respectively. Expression of the Oct2 cDNA is sufficient for
strong transcriptional activation of B-cell promoters (Müller
et al., 1988). A family of octamer binding proteins has been

described in the accompanying report (Schöler *et al.*, 1989).
Two of these proteins, namely Oct4 and Oct5, are found
in mouse oocytes. Therefore, these maternally expressed
proteins are prime candidates for regulatory factors active
in pre-implantation stages of mouse development.

In the present study, the transcriptional activity of the
octamer-motif is investigated in cells containing Oct4 and
Oct5. Oct4 is found in cells representing the germ line, such
as primary germ cells (PGC) and unfertilized oocytes, and
also in pluripotent embryonic stem cells (see Schöler *et al.*,
1989). However, Oct4 is not found in sperm and any other
tissues of the mouse. Similarly, Oct5 is detectable in extracts
of unfertilized oocytes and also in embryonic stem (ES) cells.
Oligomers of the octamer motif activate transcription in
B-cells, where Oct2 is present (Gerster *et al.*, 1987 and refs
therein). Here it is demonstrated that the octamer motif can
also strongly activate transcription in F9 and D3 stem cells
which do not contain detectable levels of Oct2 but consider-
able amounts of Oct4 and Oct5. After differentiation of these
cells, both the amount of Oct4 and Oct5 and the level of
activation driven by the octamer motif are reduced. By
microinjecting DNA into fertilized mouse oocytes, transcrip-
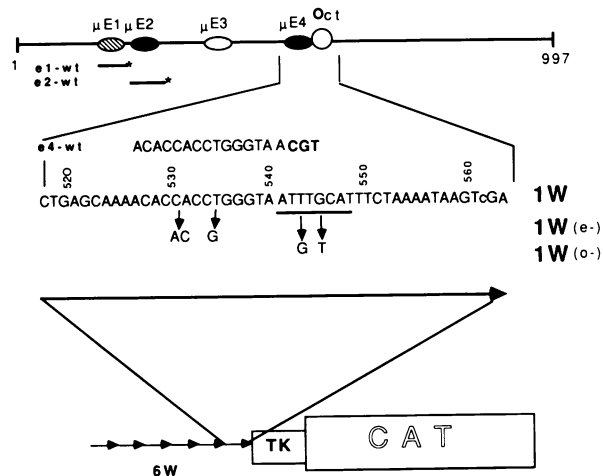

Fig. 1. Survey of the oligonucleotides used for the structural and
functional analysis of the octamer sequences in ES cells. The original
enhancer isolated as a 997 bp *XbaI*—*XbaI* fragment (Banerji *et al.*,
1983) is schematically shown with the binding sites found by genomic
sequencing (Church *et al.*, 1985; Ephrussi *et al.*, 1985). The binding
sites are represented by ellipses and a circle, designated μE1—μE4 and
Oct respectively. The 1W fragment containing the μE4 and Oct
binding sites is the smallest fragment conferring features of a B-cell-
specific enhancer (Gerster *et al.*, 1987). 1W(e-) is mutated in the μE4
binding site and 1W(o-) in the octamer motif. These mutants are
similar to those used by Gerster *et al.* (1987). e1-wt, e2-wt and e4-wt
represent the oligonucleotides that were used for μE1, μE2 and μE4
detection. The positions of the oligonucleotides in the *XbaI*—*XbaI*
fragment are indicated. To analyse the activity of the octamer motif,
1W, 1W(e-) or 1W(o-) were cloned as hexamers in front of the TK
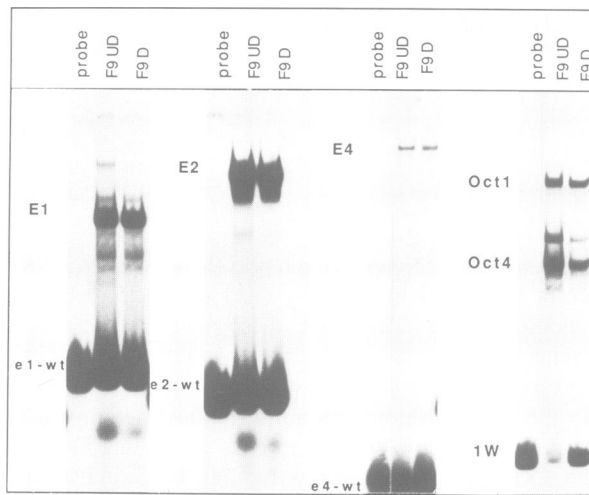promoter.

Fig. 2. Comparison of proteins binding to the octamer sequence with other sites of the heavy chain gene enhancer. Radiolabelled e1-wt, e2-wt, e4-wt or 1W oligonucleotides were incubated in the absence (probe) or presence of nuclear extracts of undifferentiated (F9 UD) or differentiated (F9 D) cells. For each lane 4 μg extract was used. e1-wt, e2-wt and e4-wt represent the oligonucleotides that were used for μE1, μE2 and μE4 detection.

Table I. Activity profile of the 6W enhancer in F9 cells

Construct	F9 UD	F9 D
pTKCAT	1	1
p6W$_s$TKCAT	>1100	55
pRSVCAT	120	130
p6W$_s$TKCAT(o-)	1	1
p6W$_s$TKCAT(e-)	750	50
pSV1CAT	<0.7	<0.7
pSV2CAT	50	80
p6W$_a$SV1CAT	90	30
p6W$_s$SV1CAT	50	nd

The results of pTKCAT, p6WTKCAT, pSV2CAT and pRSVCAT in F9 UD and F9 D were reproduced in three independent experiments with two different plasmid preparations. The other results were obtained from one experiment. ND, not done.

tional activity is demonstrated in the inner cell mass (ICM) but not in the trophoblast of blastocysts. The results suggest that Oct4 and Oct5 are maternally expressed transcription factors specifically active in early mouse embryogenesis.

Results

The octamer binding proteins decrease specifically during differentiation of F9 stem cells

Two maternally expressed octamer binding factors, Oct4 and Oct5, are found in oocytes and in D3 and F9 embryonic stem cells. When the embryonic stem cells are induced to differentiate, the amounts of the octamer binding factors decrease (Schöler *et al.*, 1989). The F9 cell line was used to define the activity of the octamer motif in the presence of Oct4 and Oct5.

F9 teratocarcinoma cells were used in many experiments to investigate early stages in mouse embryogenesis. This embryonal carcinoma (EC) cell line was established in culture from a tumour itself derived from a 6 day male

Table II. Activation of the TK promoter by 6W in cells containing Oct1

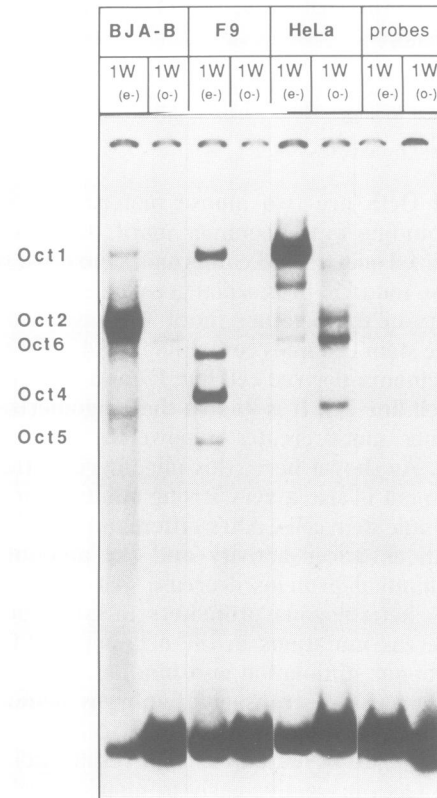| Construct	HeLa	Hep3	3T3
pTKCAT	1	1	1
p6WTKCAT	5	7	5
pRSVCAT	70	25	40

Fig. 3. Specificity of binding to the octamer motif. Radiolabelled mutations of the oligonucleotide 1W were incubated either without (probes) or with extracts of BJA-B, F9 or HeLa cells. 1W(e-) is mutated in the μE4 binding site and 1W(o-) in the octamer motif. For each lane 4 μg nuclear extract was used.

embryo transplanted into testis. F9 stem cells can be differentiated either into parietal or visceral endoderm-like cells, depending on the culture conditions (for references see Hogan *et al.*, 1986).

The octamer motif is found in a variety of promoters and enhancers (for refs see Hatzopoulos *et al.*, 1988). The octamer binding site of the immunoglobulin heavy chain gene (IgH) enhancer was used to search for octamer specific factors in mouse (Schöler *et al.*, 1989). To examine if the decrease of Oct factors during differentiation of F9 cells is specific, binding to this and other sites of the IgH enhancer (Figure 1) was compared in the electrophoretic mobility shift assay (EMSA). Three oligonucleotides with either μE1, μE2 or μE4 sites detected equal proteins amounts in undifferentiated and differentiated F9 cells (Figure 2), indicating that the difference observed is specific for the octamer binding proteins.
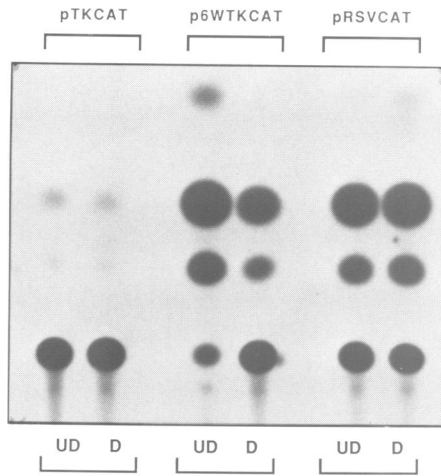
Fig. 4. Activity of the 6W enhancer in the ES cell line D3. D3 stem cells were transfected either with pTKCAT, p6WTKCAT or pRSVCAT. Twenty hours after transfection the D3 cells were either differentiated (D) or maintained undifferentiated as stem cells (UD). Sixty-eight hours after transfection the cells were harvested and used in the CAT assay. Chloramphenicol was separated from its acetylated derivatives by TLC. See also Table III.

Oligomerization of the octamer motif creates a very strong enhancer in F9 cells

Oligomerization of transcriptional motifs can create powerful enhancer elements with distinct tissue-specificities (Ondeck et al., 1987; Schirm et al., 1987; for a review see Schöler et al., 1988). In particular, oligomers of an IgH enhancer fragment containing the octamer motif (1W in Figure 1) are strong B-cell specific enhancers where Oct2 is present, but are inactive where only Oct1 is present (Gerster et al., 1987). We were interested to test if this synthetic enhancer is also active in the F9 cell line which contains the newly identified proteins Oct4 and Oct5.

An hexamer of 1W (named 6W) was cloned in front of a minimal thymidine kinase (TK) promoter whose intrinsic octamer motif had been removed (p6WTKCAT) and transfected into F9 stem cells. As positive controls plasmids containing the RSV and the SV40 enhancer/promoter regions were used (pRSVCAT and pSV2CAT). The results in Table I show that the 6W enhancer stimulates transcription at least 1100-fold as compared to the enhancer-less plasmid, although an exact determination is difficult due to the low basal activity of the TK promoter in these cells. Nevertheless, the levels of CAT activity obtained with p6WTKCAT in F9 UD cells are ~8- and ~20-fold higher than those obtained in pRSVCAT and pSV2CAT respectively. When F9 cells are induced to differentiate, the activity mediated by 6W drops 20-fold to levels below that of pRSVCAT and pSV2CAT. This reduction correlates with the decrease in binding of the octamer proteins observed in vitro.

The 6W enhancer activates different promoters
The 6W enhancer was cloned in front of the SV40 early promoter to test if the stimulation mediated by the octamer motif is conferred to different promoters. Again, transcription is stimulated by the 6W enhancer in F9 stem cells and this stimulation drops after their differentiation (Table I). Therefore the effect of the 6W enhancer is not restricted to the TK promoter. However, the 6W enhancer stimulates the

Table III. Activation of the 6W enhancer in the ES cell line D3

Construct	D3 UD	D3 D
pTKCAT	1	1
p6WTKCAT	240	45
pRSVCAT	120	140

SV40 promoter less (50- to 90-fold) than the TK promoter (1100-fold), indicating that the TK promoter is especially susceptible to transcriptional activation by the octamer motif.

To exclude the possibility that 6W becomes a ubiquitous enhancer in front of the TK promoter, this construct was tested in cell lines where only Oct1 is detectable. For this reason HeLa, 3T3 and Hep3 cells were transfected with p6WTKCAT and two reference plasmids. In each cell line 6W stimulates expression only ~5-fold (Table II) and thus the activation is much lower than in F9 stem cells. The residual activity exhibited by the TK promoter in HeLa, 3T3 and Hep3 cells is probably due to Oct1, since it has been demonstrated that Oct1 can weakly stimulate transcription in vitro (LeBowitz et al., 1988). Most importantly, these experiments make it unlikely that the activity of the 6W enhancer in F9 cells is due to Oct1.

The activity of the 6W enhancer is dependent on the octamer motif
To investigate if the stimulation in F9 cells is mediated by the octamer motif, mutations were introduced in the octamer motif and in the μE4 binding sites (Figure 1). Similar mutations have also been used to study the B-cell activity of this enhancer (Gerster et al., 1987). The mutations were first tested in the EMSA for binding and subsequently cloned in front of the TK promoter for functional analysis. The 1W(e-) binding pattern in extracts of F9, HeLa and BJA-B cells is identical to the 1W pattern, demonstrating that a mutation in the μE4 site does not affect binding to the octamer motif (Figure 3). However, the mutation in the octamer motif completely abolished binding of the Oct factors [Figure 3, 1W(o-) lanes].

Hexamers of 1W(e-) and 1W(o-) were cloned in front of the TK promoter and their activity in F9 stem cells was compared to p6WTKCAT. The octamer mutation completely abolished enhancement of the TK promoter, while altering the μE4 motif only had a minor effect (Table I). These results demonstrate that, as in B-cells, the octamer motif is the main contributor to the activity of the 6W enhancer.

6W acts as a very strong enhancer in cells derived from the inner cell mass
The F9 EC cell system has been extensively used as a model system to address a variety of questions concerning early mouse embryogenesis. However, EC cells are restricted in their developmental capacities, their exact stage of differentiation is unknown and they rarely contribute to the germ line when introduced into blastocysts (Stewart and Mintz, 1982 and refs therein). Therefore experiments were extended to ES cell lines derived from blastocysts (Evans and Kaufman, 1981; Martin, 1981) which colonize the germ line regularly and contribute to all somatic cell lineages (Bradley et al., 1984).
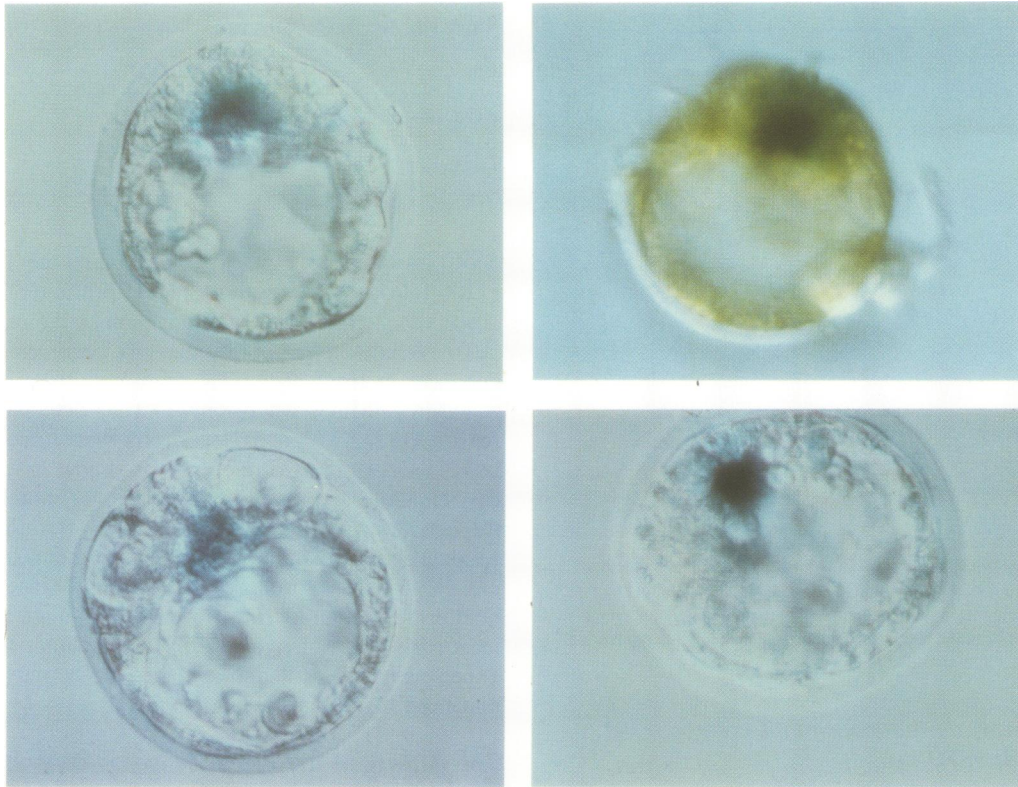
For this purpose the embryo-derived stem (ES) cell line

Fig. 5. Expression patterns of p6WTKLacZ in four independent 3.5 day blastocysts.

D3 was used (Doetschman *et al.*, 1985) which contains Oct4 and Oct5 (Schöler *et al.*, 1989). For the functional analysis of the octamer motif, p6WTKCAT was transfected into D3 stem cells in parallel with pRSVCAT and pTKCAT as positive and negative controls. As in F9 cells, the 6W enhancer was very active in D3 stem cells and, as expected from the *in vitro* results, stimulation decreases significantly after differentiation (Figure 4 and Table III, see also Schöler *et al.*, 1989). Therefore the activity of the octamer motif and the presence of the Oct factors correlate in both the ES cell line D3 and the EC cell line F9.

6WTKLacZ shows expression in the inner cell mass of blastocysts

Most experiments investigating the activity of the 6W enhancer in ES cells were performed using CAT as a reporter gene. In parallel these results were supplemented by experiments with two plasmids in which the CAT gene was replaced by *lacZ*. These plasmids−pTKLacZ and p6WTKLacZ−were transfected into F9 and D3 stem cells. As expected, the TK promoter alone gave only a small number of blue cells in both cell lines and addition of the 6W enhancer increased this number significantly (not shown). In this case the increase was ~5000-fold and therefore even higher as determined with the CAT assay.

As the ES cell line D3 had been isolated directly from 3 day blastocysts, we were interested in extending these results to the pre-implantation embryo. 6WTKLacZ and 6WTKLacZ(o-) which carries a mutation in the octamer motif as described above were microinjected into fertilized oocytes. The injected oocytes were cultured *in vitro* to the blastocyst stage or transferred to a foster mouse. After 3 days

the uteri of these mice were isolated and the blastocysts recovered.

Ninety-four oocytes were injected with 6WTKLacZ and 40 with 6WTKLacZ(o-). From these, 28 and 14 blastocysts were obtained respectively. None of the oocytes injected with 6WTKLacZ(o-) gave rise to blastocysts with detectable LacZ expression. In contrast, the transgene was active in six independent blastocysts injected with 6WTKLacZ. In five of them, activity was clearly detected in the ICM region while in one case expression was found in a few cells of the polar trophectoderm (not shown). The pattern of activity, for four blastocysts, clearly shows blue cells in the ICM (Figure 5). Interestingly, the activity seems to be restricted to only a few cells of the ICM. To visualize better this restriction, top and side views of one blastocyst are shown in Figure 6.

Discussion

Oct4 and Oct5 are maternally expressed octamer binding proteins found in the pre-implantation stages of mouse development (Schöler *et al.*, 1989). Embryonic stem cells, which contain Oct4 and Oct5, were used as a model to study the activity of the octamer in the pre-implantation embryo. So far it was assumed that oligomerization of the IgH octamer motif acts only as a strong enhancer in cells containing Oct2 (Gerster *et al.*, 1987). We demonstrate that the same enhancer, named 6W, strongly stimulates different promoters in ES cells. Therefore such an enhancer is also active in the abence of Oct2 when Oct4 and Oct5 are present. After differentiation of the stem cells, both enhancer activity and the amount of the octamer binding proteins decrease.
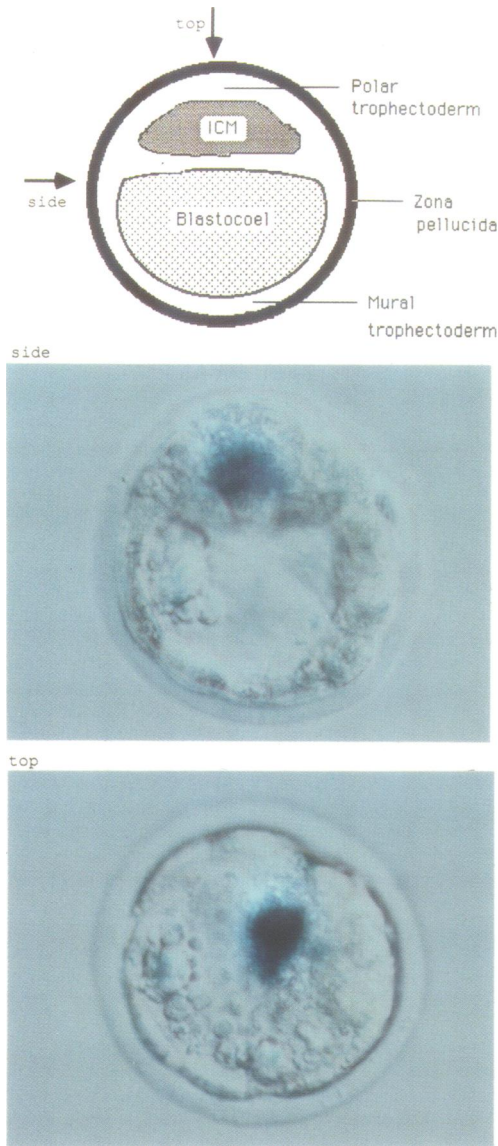
2554

Fig. 6. Expression pattern of p6WTKLacZ in a 3.5 day blastocyst. The expression of p6WTKLacZ is either shown from the side as in Figure 5 or from top.

Mutations demonstrate that the octamer motif is not only responsible for binding but is also able to stimulate transcription in the absence of any other known binding site. The pattern *in vivo* and *in vitro* correlates well, since binding of the octamer factors and transcriptional stimulation in ES cells are both abolished when the octamer motif is mutated. The biological significance of these results is demonstrated with transgenic embryos. Transgene activity was found in cells of the ICM, whereas in other cells of the blastocyst, activity was not detectable. Interestingly, the activity seems to be restricted to a few cells of the ICM.

Although octamer binding proteins obviously play a role in transcriptional stimulation, it is not possible to attribute this function to a single protein found in embryonic stem cells. Oct4 is a prime candidate for transcriptional stimulation since this factor gives the most prominent complex with ES cell extracts. The intensity of the Oct4 complex in extracts of F9 cells is almost as high as that of Oct2 in BJA-B cells (Schöler *et al.*, 1989). Although much less abundant, a

stimulation of the enhancer activity could also be due to Oct5 or Oct6 since high amounts of complexes do not necessarily correlate with stimulation.

The high activity of the 6W enhancer in these cells is presumably not due to Oct1. The amount of Oct1 decreases only slightly after differentiation and cannot explain the dramatic decrease of transcriptional activity observed *in vivo*. Moreover, transfection experiments in cell lines where only Oct1 is detectable show that the 6W enhancer is at least two orders of magnitude less active than in cells containing other octamer binding proteins. In this respect, ES cells are similar to B-cells in which the same enhancer strongly stimulates transcription due to Oct2 (Gerster *et al.*, 1987). It is possible though that Oct1 acts synergistically with either Oct4 and/or Oct5 to stimulate transcription.

The results presented here are in apparent contradiction with previously published data. Fromental *et al.* (1988) could not demonstrate any activity of the SV40 octamer in F9 cells although the same constructs are active in B-cells. This particular arrangement of the SV40 octamer sequence motifs in different enhancers might be unfavourable for binding of the factors found in F9 cells. As shown in the accompanying paper (Schöler *et al.*, 1989), binding of the factors is strongly affected when the surrounding sequences of the IgH octamer are altered.

In a recent publication, the octamer motif of the IgH enhancer repressed transcription in F9 stem cells. In this study, an IgH fragment harbouring the μE3 site in addition to the μE4 and octamer sites was placed in front of a truncated promoter of the *fos* gene (Lenardo *et al.*, 1989). The activity of this fragment was compared with that of mutations in the different motifs. Whenever an intact octamer motif was present, μE3 could not stimulate the *fos* promoter. Only when the octamer motif was mutated could a weak activity be demonstrated for the μE3 motif. Thus it appears that the *fos* promoter is not directly influenced by the octamer sequences but that the effect of the μE3 motif is blocked by the binding of proteins to the octamer motif.

These apparently contradictory results could be explained as follows. In the case of the 6W enhancer the arrangement of the various motifs might allow protein binding without steric hindrance, thereby enabling the octamer protein(s) to participate actively in a transcriptional complex. In the case of the *fos* promoter/IgH enhancer constructs, the motifs might be placed in such a way that binding of the octamer protein(s) would hinder formation of a transcriptional complex. This explanation is not unlikely since a similar effect of the octamer motif has been previously demonstrated (Parslow *et al.*, 1987). In this study the TK promoter containing the octamer motif was placed under the influence of the Ig$\varkappa$ light chain and the MSV enhancer. When the Ig$\varkappa$ enhancer was used in B-cells, mutations in the octamer reduced activity. If the MSV enhancer was used, then mutations in the octamer motif increased transcriptional enhancement. These findings strengthen the presumption that the particular context in which a transcription motif is found can influence its properties.

Another explanation for the contradictory results is that the inhibitory effect described by Lenardo *et al.* (1989) is due to one Oct factor and the strong transcriptional stimulation is due to another. Supporting this idea, the ratios of Oct4 and Oct5 are inverse in oocytes and ES cells. Oct4 and Oct5 are the most prominent octamer binding proteins

in oocytes and ES cells respectively (Schöler et al., 1989). If Oct5 acts as a repressor, a decrease of this maternal protein would relieve repression of certain genes during development. Simultaneously, Oct4 could stimulate genes in the pre-implantation embryo.

Although oligomerization has been shown to be a powerful method emphasizing the effects of regulatory elements (for discussion and refs see Schöler et al., 1988), it has to be stated that an oligomerized unit reflects an artificial situation that may have unexpected properties. Therefore it is necessary to study genes that are regulated by the octamer motif in early embryogenesis.

In contrast to our findings, Gorman et al. (1985) did not observe transcriptional stimulation in F9 cells mediated by the SV40 enhancer. They concluded that an activity repressing the SV40 enhancer is present in these cells. The octamer motif of the SV40 enhancer is a possible target of this repressing activity, since an inhibitory effect mediated by the octamer motif in F9 cells had been suggested by Lenardo et al. (1989). To explain the stimulation in our experiments, we assumed that the repressing activity was ineffective due to competition by high amounts of transfected DNA. To test this assumption, different amounts of pSV1CAT and pSV2CAT were used adding carrier DNA to same total amounts (not shown). However, at low amounts of specific DNA, stimulation was even higher since the activity of pSV1CAT increased linearly whereas the activity of pSV2CAT reached a plateau at low amounts. This indicates the presence of a limiting amount of activating factor(s) binding to the SV40 enhancer but cannot confirm a repressing activity. The Oct factors were not limited since the 6W enhancer activity increased steadily (not shown). In agreement with us, Sassone-Corsi et al. (1985) reported a 30-fold stimulation of the rabbit β-globin promoter and Nomiyama et al. (1987) observed a 100-fold stimulation of the SV40 promoter in F9 stem cells.

During the first stages of mouse development the blastomeres of the embryo are considered to be equipotent. Between the early 8-cell stage and the 16-cell stage the developmental potency of the cells becomes restricted. This results in the generation of two distinct lineages in the 16-cell morula: one or two internal cells give rise to the ICM, whereas most of the descendants of the external cells become trophectoderm. The trophectoderm develops to the extra-embryonic chorion, the outer portion of the placenta, whereas the ICM gives rise to the embryo. When pre-implantation embryos were investigated, in five out of six embryos expressing the transgene, the oligomerized octamer motif activated expression in the ICM but not in the trophoblast. Moreover, transcription seems to be restricted to certain cells of the ICM. This would be the first indication that cells in the ICM are different. However, although five independent blastocysts show the same pattern, we cannot exclude at this point that the restriction in the ICM is due to mosaicism of the transgenic blastocysts or some other effects. To prove the exciting prospect of restricted expression in the ICM, we are currently establishing transgenic mouse lines carrying the LacZ construct under the influence of the 6W enhancer.

The intriguing finding that Oct4 is specifically present in embryonic stem cells has many implications for control processes during early stages of development. It will be interesting to determine if the Oct factors present in pre-

implantation stages of the mouse regulate members of the Hox or Pax gene families. The strong activity of 6W in undifferentiated embryonic stem cells might render this enhancer a useful tool for driving high expression levels of selected genes during early development. Further studies will be directed towards the identification of the target genes that are regulated by Oct4 and Oct5 during mouse embryogenesis.

Materials and methods

Cell lines and transfection
F9, 3T3, Hep3 (human hepatoma), BJA-B and HeLa cells were grown as described previously (Schöler and Gruss, 1984, 1985). D3 cells were cultured as described by propagating them in highly purified, recombinant LIF to maintain the undifferentiated phenotype (Williams et al., 1988b). The F9 (UD) cells were differentiated to F9 (D) by treating them 48 h before harvest with 5×10^{-7} M retinoic acid and 10^{-3} M cAMP. To differentiate D3 cells, LIF was omitted and both retinoic acid and dibutyryl cAMP were added to final concentrations of 10^{-7} and 10^{-3} M respectively (Doetschman et al., 1985; Williams et al., 1988a).

The F9 cells were transfected as described elsewhere (Schöler and Gruss, 1984) with one modification. Instead of treating the F9 cells with a glycerol shock, the calcium phosphate precipitate was left on the cells for 20 h. After 20 h the medium of the F9 cells was either replaced to maintain the undifferentiated state of the cells or was changed to medium containing 5×10^{-7} M retinoic acid/10^{-3} M dibutyryl cAMP to yield differentiated cells (Strickland et al., 1980). The cells were harvested 48 h after changing the medium.

The undifferentiated D3 cells were transfected as described elsewhere (Schöler and Gruss, 1984), with the exception that 5 cm dishes were used and the DNA−calcium phosphate precipitate was left on the cells for 20 h. The cells were subsequently trypsinized and plated on two 10 cm tissue culture dishes and maintained for 48 h in medium with or without LIF (see above).

CAT assays were performed as described elsewhere (Schöler and Gruss, 1984) with one modification: after sonication the complete extract was incubated for 10 min at 60°C to denature proteases and afterwards cooled on ice.

LacZ detection in blastocysts and in cultured cells
Fertilized oocytes were obtained from outbred NMRI mice. All procedures to obtain the microinjected blastocysts are essentially as described by Hogan et al. (1986). The blastocysts were first washed twice in PBS and fixed in 0.25% glutaraldehyde at 4°C for staining. After 10 min they were washed four times in PBS and stained in a solution of 1 mg/ml X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$ and 2 mM $MgCl_2$ in PBS.

The procedure for LacZ detection in cultured cells is as described for blastocysts, except that cells were fixed in methanol.

Preparation of nuclear extracts
Nuclear extracts of the cell lines were prepared according to the protocol of Dignam et al. (1983). Nuclear extracts from mouse tissues and embryos were prepared according to the method of Gorski et al. (1986) including a mixture of protease inhibitors: PMSF (0.5 mM), leupeptin (0.5 μg/ml), pepstatin (0.7 μg/ml), aprotinin (1 μg/ml) and bestatin (40 μg/ml) in the homogenization buffer.

Electrophoretic mobility shift assay (EMSA)
Binding conditions for the EMSA were 10 mM Hepes, pH 7.8 (4°C), 1 mM spermidine, 5 mM $MgCl_2$, 50 mM KCl, 0.5 mM DTT, 9% glycerol, 1 μg polyd(I·C), 10 000 c.p.m. of the labelled oligonucleotide. The final reaction volume was 15 μl. After an incubation for 15 min at 25°C the binding reaction was applied to gel electrophoresis as described by Singh et al. (1986).

Oligonucleotides
The numbering of all oligonucleotides is according to Ephrussi et al. (1985). 1W is identical to region 518−564 except for position 562 (Gerster et al., 1987). In 1W(e-) the 1W oligonucleotide is changed at positions 531, 532 and 535 to A, C and G respectively. In 1W(o-) the 1W oligonucleotide is changed at the positions 543 and 545 to G and T. The octamer motif (541−548) is underlined. e1-wt (334−380), e2-wt (385−417) and e4-wt

(527−541) are identical to the regions containing binding sites found by genomic footprinting of B-cells (Church *et al.*, 1985; Ephrussi *et al.*, 1985). The sequences of the coding strands of 1W and e4-wt are shown.

Recombinant plasmids

Construction of p6W$_s$TKCAT, p6W$_s$SV1CAT and p6W$_a$SV1CAT: the *Eco*RI−*Eco*RI fragment of the FD hexamer (Gerster *et al.*, 1987), referred to here as 6W, was first cloned into the *Eco*RI site of the Bluescript polylinker. Using the *Xba*I and the *Hin*dIII sites of the vector polylinker the 6W fragment was cloned in the same sites of pTKCAT. Fragments carrying mutated motifs were similarly cloned. pTKCAT is derived from pBLCAT2 (Luckow and Schütz, 1987) by inserting two SV40 termination signals in front of the pBLCAT2 polylinker to decrease unspecific transcription (M.Koenen, personal communication). The position of the termination signal in the SV40 genome is 2533 (*Bam*HI) to 2770 (*Sau*3A) according to Tooze (1981). The octamer motif, which is an intrinsic part of the TK promoter, is not present in pTKCAT (Luckow and Schütz, 1987; Parslow *et al.*, 1987). For p6W$_s$SV1CAT and p6W$_a$SV1CAT, the 6W fragment was isolated from the Bluescript construct by cutting the polylinker with *Bam*HI and *Bgl*II and recloning the fragment in both orientations 5′ of the SV40 promoter in pSV1CAT. pSV1CAT, pSV2CAT and pRSVCAT are described by Gorman *et al.* (1985). p6WTKLacZ, pTKLacZ and p6WTKLacZ(o-) were constructed by replacing the CAT gene of the respective CAT plasmids by a *Hin*dIII−*Bam*HI fragment containing the *lacZ* gene. The LacZ fragment was derived from pCH110 (Pharmacia). The 4 kb fragment used for microinjection was isolated after restricting the 7 kb plasmid with *Sph*I and *Sac*II.

Acknowledgements

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