17. Maden, M., Ong, D. E., Summerbell, D. & Chytil, F. *Development* **107** *Supplement* 109–119 (1989).
18. Jessell, T. M., Bovolenta, P., Ptaczek, M., Tessier-Lavigne, M. & Dodd, J. *CIBA Fdn. Symp.* **144**, 255–280 (1989).
19. O'Brochta, D. & Bryant, P. *Nature* **313**, 138–141 (1985).
20. Patel, N. H. *et al. Cell* **58**, 955–968 (1989).
21. Wilkinson, D. G., Bailes, J. A. & McMahon, A. P. *Cell* **50**, 79–88 (1987).
22. Fujita, S. *Expl Cell Res.* **28**, 52–60 (1962).

ACKNOWLEDGEMENTS. This study was supported by grants from the NIH to S.F., from the MRC, the Royal Society and the Wellcome Trust to R.J.K. and A.L., and from the Dickinson Trust to A.L.

New type of POU domain in germ line-specific protein Oct-4

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MEMBERS of a family of murine octamer-binding proteins interact specifically with the octamer motif, a transcription regulatory element found in the promoter and enhancer regions of many genes[1]. Oct-4 is a maternally expressed protein that is also present in the pre-implantation mouse embryo[1,2]. Although many regulatory proteins are expressed in post-implantation embryos[3], transcription factors regulating pre-implantation processes have remained elusive. The *Oct-4* gene is therefore a prime candidate for an early developmental control gene. Here we report the complementary DNA cloning of the mouse *Oct-4* gene, and the characterization of the encoded protein(s) by sequential *in vitro* transcription, translation, DNA-binding and protease-clipping analysis. Deletion analysis shows that the DNA-binding activity is mediated by a POU domain encoded in an open reading frame corresponding to a 324-amino-acid protein[4–13]. Sequence comparison with known POU domains reveals that Oct-4 is a novel member of the POU-family.

The proteins Oct-1 and Oct-2 both contain a POU domain[6–10] involved in DNA-binding at octamer motifs[14]. Because Oct-4 binds to the same motif as do Oct-1 and Oct-2, we used a probe spanning the POU domain of the mouse *Oct-2* gene to screen a cDNA library prepared from F9 stem cells which contain Oct-4 in abundance (see Fig. 2 legend)[1,15]. The inserts of 18 positive recombinant phages were transcribed *in vitro* and then translated in a reticulocyte lysate. The translation products were tested for DNA-binding in an electrophoretic mobility shift assay (EMSA) using an oligonucleotide probe containing the octamer motif (probe 1W; Fig. 1).

The products of one clone (which we will refer to as the juhachi clone) shifted the 1W probe to the position of Oct-4 and Oct-5, as found in F9 nuclear extracts (Fig. 1*a*, lanes 1 and 2). We performed proteolytic clipping experiments to test the relationship of the juhachi-clone products with Oct-4[1,16]. Reticulocyte extracts containing both the translation products of either the juhachi clone or an *Oct-1* clone (see Fig. 1*a*) and a nuclear extract of F9 cells, were incubated with probe 1W and then digested with different amounts of a nonspecific endoprotease (Fig. 1*b*, Oct-1 clipping not shown). A comparison of the proteolytic intermediates showed that the pattern with the juhachi-clone gene products is identical to that of Oct-4, with the exception of minor bands most likely resulting from Oct-1 degradation in the F9 extract (Fig. 1*b*, *c*). The degradation pattern obtained with the F9 extract seems to be a combination of those of the cloned Oct-1 and Oct-4 proteins (Fig. 1*c*). The position of the juhachi-clone translation products in the EMSA, and the degradation pattern, strongly indicate that the juhachi clone encodes Oct-4.

Sequence analysis of the cDNA identifies an open reading frame encoding 324-amino-acid protein (Fig. 2*a*). Six clones yielding smaller octamer-binding proteins (see legend of Fig. 1*a*) represent incomplete 5′ ends of the juhachi clone (clones 1 and 16 of Fig. 2*a*). Comparison of the sequence of Oct-4 with the sequences of POU domain-containing proteins[4–13,17] reveals a 79-amino-acid region that is homologous to the POU-specific domain, and a 60-amino-acid region homologous to the POU homoeodomain (Fig. 2*a*, *c*). The two regions are separated by a stretch of 17 amino acids which has no homology to any other POU domain-containing protein. Similar to other homoeodomains, that of Oct-4 is predicted to contain four helices, with a cluster of basic amino acids preceding the first helix (cluster I) and a cluster overlapping the end of the third

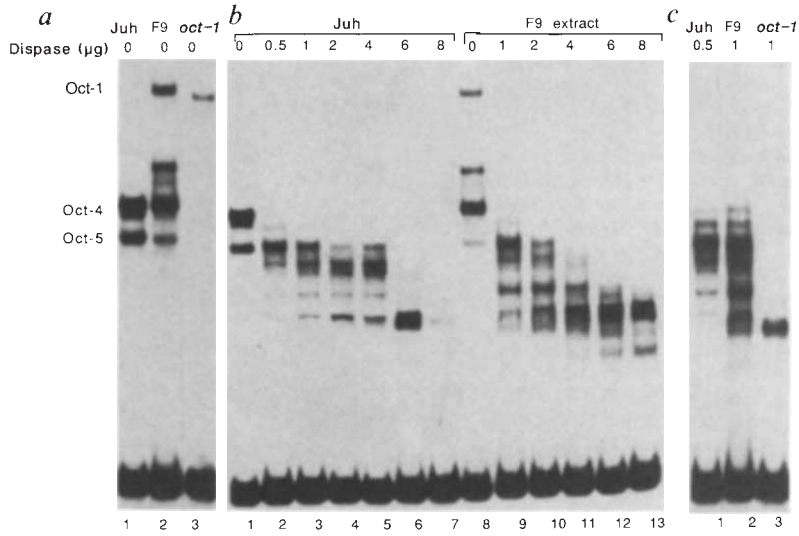
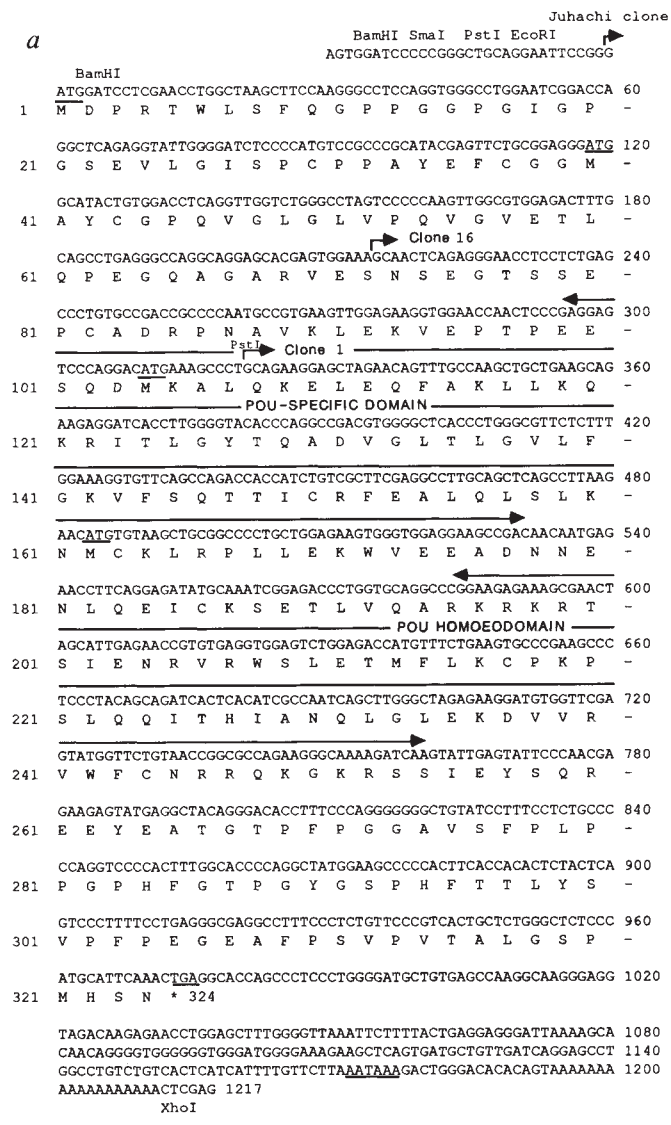


FIG. 1 Identification of the F9 *Oct-4* cDNA clone by EMSA and proteolytic clipping. *a*, Comparison of the juhachi clone (Juh; lane 1) and a mouse *Oct-1* clone (lane 3) with F9 octamer-binding proteins (lane 2). Translation products of one group of clones (results for only one are shown) formed a complex migrating slightly ahead of the mouse Oct-1 complex (compare lanes 2 and 3). These clones were identified by sequence analyses as mouse homologues of, and having the same 5′ ends as, the human *oct-1* gene[7], indicating that the difference in migration is probably due to post-translational modifications. A second group of clones yielded DNA-binding products either at the position of Oct-4 and Oct-5. (Juk; lane 1) or smaller (data not shown). The smaller products were identified as 5′ deleted juhachi clones. A third group of clones not related to these groups was investigated separately (data not shown). *b*, Proteolytic clipping EMSA of the gene products of the juhachi clone (lanes 1–7) and F9 octamer-binding proteins (lanes 8–13). The proteins were treated without (lanes 1 and 8) or with different amounts of dispase as indicated. *c*, Comparison of octamer-binding intermediates obtained by proteolytic clipping of the translation products of the juhachi clone (lane 1) and an *Oct-1* clone (lane 3) with proteolytically degraded octamer-binding proteins of F9 cells (lane 2). Lanes 1 and 2 represent longer exposures of lanes 2 and 9 in *b*, respectively. METHODS. Clone 18 (juhachi clone) and clone 3 (*Oct-1* clone) were linearized at the unique *Xho*I restriction sites (added at the 3′ end of the cDNAs because of the directional cDNA cloning procedure) and purified with Biogel P30 (Biorad) spin-columns. RNA was synthesized with T3 RNA polymerase (Promega) using 50 U μg^{-1} DNA. Reaction conditions, 40 mM Tris–HCl buffer, pH 8.0, 8 mM $MgCl_2$, 25 mM NaCl, 2 mM spermidine, 5 mM DTT, 2 mM ATP, 2 mM 6TP, 2 mM UTP, 2 mM CTP and 2 mM $m^7G(5')ppp5'G$. An aliquot of the transcription mixture containing about 1 μg RNA was added to a rabbit reticulocyte lysate (BRL) in accordance with the manufacturer's specifications. The translation products were analysed in parallel by SDS-PAGE (data not shown) and tested for binding in an EMSA. Translation assay (1 μl) was used for the EMSA as described[1], except the amount of poly d(I·C) was reduced to 0.4 μg. The F9 nuclear extract was prepared as described[1]. The EMSA was performed with probe 1W containing the octamer of the enhancer of the mouse immunoglobulin heavy chain gene[1].

FIG. 2 Nucleotide sequence of F9 *Oct-4* cDNA and deduced amino-acid sequence of the Oct-4 protein. *a*, Numbering (right, nucleotide sequence; left, amino-acid sequence in one-letter code) begins at the putative initiation codon. The POU-specific domain and the POU-homeoeodomain are marked above the nucleotide sequence. The arrows and numbers of clones indicate positions of 5′ cDNA deletion mutants. The four methionines corresponding to putative initiation codons of different clones are underlined. Each of the codons are in a favourable context[27] and the corresponding proteins were confirmed by SDS-PAGE. Stop codon TGA and consensus polyadenylation hexamer AATAAA are underlined. Linker sequences 5′ and 3′ of the juhachi clone are included. *b*, Expression of *Oct-4* sequences in F9 stem cells as detected by northern-blot analysis. The blot containing F9 poly(A)$^+$ RNA was hybridized to an *Oct-4* probe spanning the 5′ region of the gene. The positions and lengths of the RNA marker are indicated. *c*, Predicted amino-acid sequence (single-letter code) of Oct-4. The POU-specific domain, the POU homoeodomain with the four predicted helices, basic clusters (thick bars), and hinge region between helices 1 and 2 are indicated. ⊖, Acidic amino acids; *hexa* and *tri*, hexamer and trimer sequences between the predicted helices[20]. *d*, Percentage homology of Oct-4 POU domain to different members (underlined) of the POU domain classes[11]. The similarity to the POU-specific domains A and B (ref. 17), the POU-homoeodomain and to each predicted helix and the hinge is presented.

METHODS. Cloning: Total RNA was isolated from F9 stem cells by the guanidinium thiocyanate method[28]. Poly(A)$^+$ RNAs were prepared by retention on oligo(dT) columns. A directional cloned cDNA library in the λ expression vector λZAPII (Stratagene) was constructed essentially as described[29] with the following modifications: single-stranded cDNA primed with the oligonucleotide 5′-AATTCCTCGAG(T)$_{15}$-3′ containing a *Xho*I restriction site, was prepared using avian myeloblastosis virus (AMV) reverse transcriptase (Boehringer) and 5-methylcytosine. Double-stranded cDNAs, methylated at internal *Eco*RI sites was ligated to 12mer *Eco*RI linkers (CCGAATTCCGG, Boehringer). After recutting with *Eco*RI and *Xho*I, and size fractionation on a Biogel A50m column (Biorad), the cDNA inserts were ligated to *Eco*RI/*Xho*I-digested λZAPII vector DNA. After *in vitro* packaging, the phages were plated on *E. coli* PLK-F′ (Stratagene). The average insert size was 2.1 kb (0.7–7.6 kb, based on 34 phage 'minipreps'). Screening: for isolation of λ phage recombinants containing POU-related sequences, 10^6 independent plaques were screened using Hybond membranes (Amersham). The DNA membranes were baked for 2 h at 80 °C. Prehybridization was at 60 °C in 7 × SSC, 5 × Denhardt's solution, 0.5% SDS, 0.1 mg ml^{-1} denatured salmon sperm DNA; hybridization was performed in the same solution containing 10^6 c.p.m. ml^{-1} of a 0.6-kb mouse *Oct-2* probe spanning the POU domain (A. Hatzopoulos, unpublished results) at 65 °C for 16 h. The filters were washed in 1 × SSC, 0.1% SDS, at 25 °C. The inserts were then excised by a helper phage to generate Bluescript II SK recombinant phagemids for sequencing and *in vitro* transcription. Sequencing was performed with the dideoxy chain termination method using single-stranded and double-stranded DNA. (Sequenase Kit, USB). For northern-blot analysis, poly(A)$^+$ RNA was isolated as described above. About 5 μg was electrophoresed through a 1% agarose gel in 3.7% formamide and MOPS buffer (20 mM morpholine propane sulphonic acid, 50 mM sodium acetate, 10 mM EDTA, pH 7.0). After electrophoresis, the gel was soaked in 20 × SSC for 30 min and blotted onto Genescreen-plus (NEN-Dupont) overnight with 10 × SSC. The filter was baked at 80 °C for 2 h and hybridized overnight with a *Pst*I–*Pst*I *Oct-4* probe (10^6 c.p.m. ml^{-1}) at 42 °C in 50% formamide, 1 M NaCl, 1% SDS and 100 μg ml^{-1} denatured salmon sperm DNA. The blot was washed twice for 15 min in 2 × SSC, 1% SDS, and then washed in 0.1 × SSC, 1% SDS at 60 °C. The autoradiograph was exposed overnight.



d

POU class	Members	POU-specific subdomain A	POU-specific subdomain B	POU homoeodomain Complete	Helix 1	Hinge	Helix 2	Helix 3
I	Pit-1	58	74	57	23	50	36	91
II	Oct-1; Oct-2	**73**	79	58	46	50	55	82
III	SCIP (Tst-1) Brn-1; Brn-2	**73**	**88**	65	46	**100**	36	91
	Ceh-6	69	**91**	63	46	37	45	91
IV	Unc-86, Brn-3	61	56	48	23	50	36	82

helix (cluster II)[18,19] and most of the fourth helix[20]. Another region rich in basic amino acids is located in the centre of subdomain A of the POU-specific domain (cluster III). The regions outside the POU domain are rich in proline residues, interspersed with acidic amino acids at the N-terminal region of the protein. Transcriptional activation by this acidic region, as has been described for other proteins[21], remains to be shown.

The POU domain of Oct-4 is different from all classes of POU domains described so far[11]; it has the highest homology to class III POU domains (Fig. 2*d*). Two regions of homology between the POU domain of Oct-4 and class III POU domains are especially striking: subdomain B of the POU-specific domain[17], and the possible hinge region between helices 1 and 2 of the POU homoeodomain[20]. Subdomain B is identical at 30 of 34 positions to rat SCIP (Tst-1)[11,13], and at 31 positions to the *Caenorhabditis elegans* protein Ceh-6 (ref. 12). Moreover, two of the remaining amino acids represent conservative changes (Leu–Val; Lys–Arg). In the hinge region, 100% identity to SCIP (Tst-1) is found, although the flanking helices show only about 40% homology. This homology could reflect an interaction of SCIP (Tst-1) and Oct-4 with a common target. It is interesting that although SCIP (Tst-1) and Ceh-6 are highly homologous members of class III POU domain-containing proteins their homology in the hinge region is only 37%. The highest similarity between all classes and Oct-4 is found in the predicted recognition helix (helix 3). Only one substantial change in this helix is found between Oct-4 and the members of class IV proteins.

A 1.6-kilobase (kb) transcript was detected in F9 stem-cell RNA using an *Oct-4*-specific probe (Fig. 1*b*). No *Oct-4* transcript could be detected in RNAs of day-12.5 embryos, testis, liver, lung, intestine, brain or kidney (results not shown). This is support for the expression of *Oct-4* being strongly developmentally regulated[1,2].

The use of two in-frame ATG codons from one transcript to generate different products has been predicted for a variety of proteins, including some oncogenes, hormone receptors and growth factors[22]. The two translation products produced by the juhachi-clone RNA (see Fig. 1*a*, lane 1) could also result from the use of the two ATG codons at the 5′ end of this clone. To test this possibility, we deleted the first ATG codon. Translation of the resulting RNA produced a protein that migrated to the Oct-5 position in the EMSA, showing that both codons can be used for initiation (Fig. 3*a*). Although the similarity in size of the smaller translation product and Oct-5 is highly suggestive, it remains to be demonstrated whether Oct-5 is indeed translated from the *Oct-4* RNA *in vivo*. Alternatively, the production of differential splicing products with or without the first AUG could also give rise to both proteins. If such post-transcriptional regulation occurs, the different ratios of Oct-4 to Oct-5 in F9 cells and unfertilized oocytes[1] would then indicate that the expression of these proteins is differentially regulated during mouse development. Indeed, differential expression of translational initiation can be mimicked *in vitro* (Fig. 3*b*).

We examined the DNA-binding domain by using two of the 5′ deletions of the *Oct-4* gene and further deletions using suitable restriction sites. Analysis of the different 5′ deletions reveals that binding still occurs when the POU-specific domain is nearly deleted. The shorter protein encoded by clone 1, initiating translation at the C-terminal half of the POU subdomain B, can still bind to DNA (Fig. 4, B in *a* (lane 5) and *c*). But when the same clones were restricted with *Bgl*I or *Ban*I before *in vitro* translation to remove the C terminus[23–25], the clones with lesser 5′ deletions encoded proteins that still bound DNA, whereas the protein encoded by the clone with the largest 5′ deletion (clone 1) completely lost DNA-binding activity (Fig. 4*a*, lane 6). This deletion of the C terminus removes three of the five basic amino acids of cluster II (Fig. 4*c*). Because shortened translation products that retain the complete POU domain form DNA complexes, sequences in the POU-specific domain must contribute to DNA-binding. In addition, because the translation

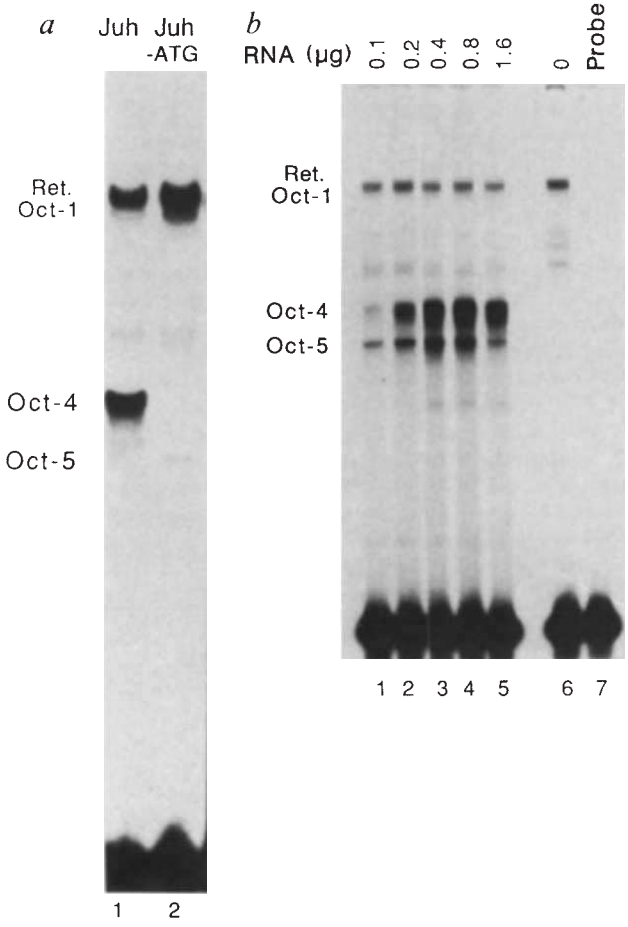


FIG. 3 *In vitro* translation of Oct-4 and Oct-5. *a*, Examination of initiation codon use in the juhachi clone (Juh). The translation products of the juhachi clone (lane 1) and a 5′-deleted clone without the first ATG (lane 2) are compared. The ATG was deleted by cutting the juhachi clone with *Bam*HI and then religating. One *Bam*HI site resides in the polylinker upstream of the gene, the other overlaps with the first ATG codon (Fig. 2*a*). The positions of Oct-4 and Oct-5 are indicated. *b*, Differential *in vitro* translation of Oct-4 and Oct-5. Different amounts of the *in vitro* transcription assay containing juhachi clone RNA were incubated with the reticulocyte lysate. The amounts of RNA are indicated above lanes 1–5. Lane 6, extract without RNA; lane 7, probe. Ret.Oct-1 is Oct-1 derived from the reticulocyte lysate.

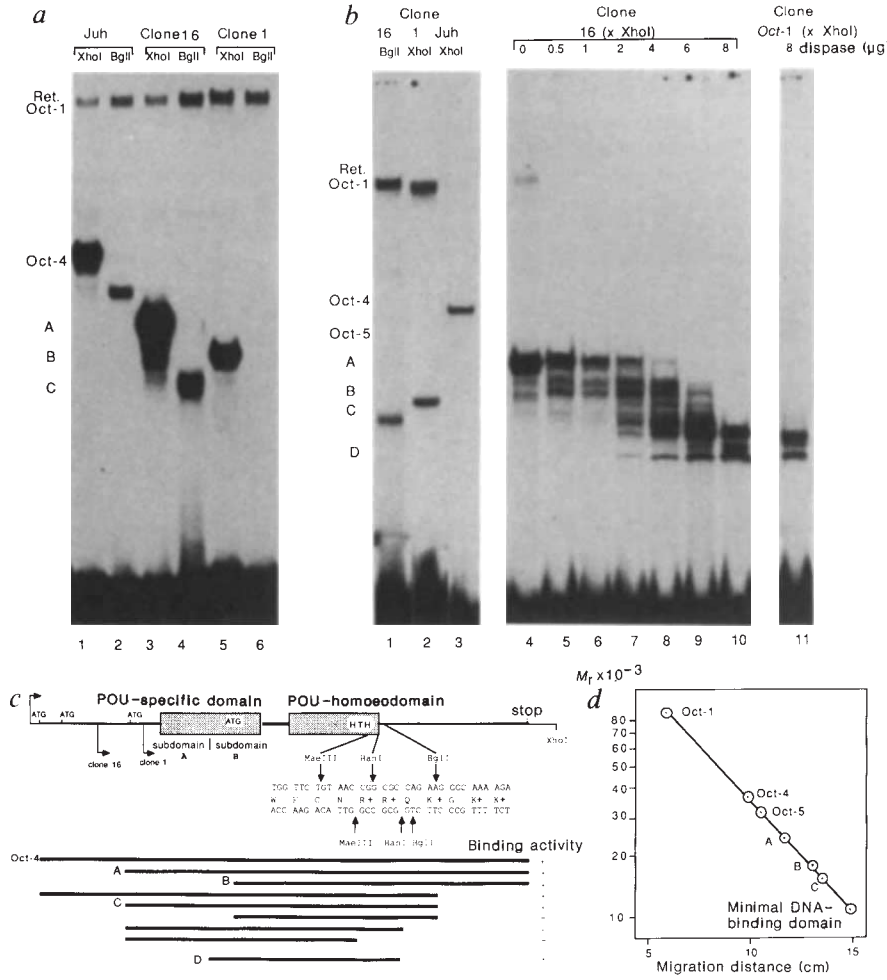


FIG. 4 Delineation of the DNA-binding domain. *a*, Determination of the C-terminal border of Oct-4 DNA-binding domain. The juhachi clone (Juh; lanes 1, 2), clone 16 (lanes 3, 4) and clone 1 (lanes 5, 6) were cut either with *Xho*I (lanes 1, 3, 5), *Bgl*I (lanes 2, 4, 6), *Ban*I (data not shown) or *Mae*III (data not shown) before expression and EMSA. In parallel, ^{35}S-labelled translation products were analysed on an SDS-PAGE showing slightly greater amounts of the clone-16 translation products (data not shown). *b*, Estimation of the N-terminal border of Oct-4. The translation products produced by restriction of the different clones (lanes 1–4) are shown in parallel with the degradation intermediates of the clone-16 translation product (lanes 4–10) and the final detectable products of an Oct-1 protein (lane 11). To use the translation products as length markers, different amounts of protein were applied to the EMSA. Therefore the intensities of the complexes do not reflect DNA-binding affinity. Ret. Oct-1 in *a* and *b* is Oct-1 derived from the reticulocyte lysate. *c*, Schematic representation of the Oct-4 protein indicating corresponding positions of 5′ deletions and restriction sites used for protein mapping. The C-terminal border of the homoeodomain is enlarged to show the nucleotide sequence, the deduced amino-acid sequence (single-letter code) and the restriction sites used in *a*. The lengths of the respective proteins and their binding activity are shown below. A–D, translation products of the different clones or their shorter counterparts. *d*, Length calibration of the minimal Oct-4 DNA-binding domain by extrapolation[16] using the M_rs of the products shown in lanes 1–4 of Fig. 4*b* (see text).

product of clone 1 which is missing most of the POU-specific domain can still bind to DNA, sequences C-terminal to the POU homoeodomain must also contribute to DNA-binding. Therefore, stable complex formation is possible when at least one of these regions is present.

To determine the minimal DNA-binding domain of Oct-4, the EMSA with the deletion mutants was combined with the proteolytic clipping assay[1,16]. The *Ban*I site marks the C-terminal border of the minimal DNA-binding domain, because any further removal of amino acids abolished stable binding (*Mae*III restriction in Fig. 4*c*). Moreover, proteolytic clipping of the complexes with the full-length or the shorter translation products of clone 16 and clone 1 gave the same final complexes (Fig. 4*b*, only clone 16 restricted with *Xho*I is shown). To determine the approximate size of the minimal DNA-binding domain, the different translation products were used as markers (Fig. 4*b*, lanes 1–4) and their calculated relative molecular masses (M_r) were plotted against the distances of migration on a logarithmic scale (Fig. 4*d*). The M_r of the minimum binding protein was ~11,000, corresponding to 100 amino acids as deduced by extrapolation. According to this calculation, the N-terminal border would coincide with the border between subdomains A and B of the POU-specific region (Fig. 4*c*).

Oct-4 is present in primordial germ cells, unfertilized oocytes and in the inner cell mass of the mouse blastocyst[1]. Therefore, this putative transcription factor could act at the very top of the hypothetical cascade of control events. The studies described here show that Oct-4 contains a new type of POU domain (class V). Although it is necessary for DNA-binding, this domain could also function by interacting with other proteins. Two sequences highly conserved between the POU domain of Oct-4 and class III POU domains are good candidates for further studies. We believe that our results will help in the study of the function of Oct-4 during murine embryogenesis.

Note added in proof: After this manuscript was submitted, Okamoto *et al.*[30] described the cDNA cloning of an octamer-binding protein (Oct-3; from P19 cells) that is probably also encoded by the Oct-4 gene. There are two differences between the P19 and the F9 cDNA clones. First, a difference at the C-terminus that can be explained by an additional G in our sequence at position 800 which results in a frameshift. We do not think that this additional G is due to the cDNA cloning procedure. Without this frameshift Oct-4 and Oct-5 would be 25 amino acids longer. Such a difference would be detectable in the EMSA. However, Oct-4 and Oct-5 expressed from the Oct-4 cDNA clone are at the same position as F9 Oct-4 and Oct-5. Second, the Oct-4 and Oct-3 cDNA clones have different 5′ sequences, possibly due to differential splicing. This difference results in a N-terminal replacement of the MetAspPro sequence in Oct-4 by 31 different amino acids in Oct-3. The differences between Oct-4 and Oct-3 do not affect the POU domain. □

Received 24 October 1989; accepted 5 February 1990.

1. Schöler, H. R., Hatzopoulos, A. K., Balling, R., Suzuki, N. & Gruss, P. *EMBO J.* **8,** 2543–2550 (1989).
2. Schöler, H. R., Balling, R., Hatzopoulos, A. K., Suzuki, N. & Gruss, P. *EMBO J.* **8,** 2551–2557 (1989).
3. Holland, P. W. H. & Hogan, B. L. M. *Genes Dev.* **2,** 773–782 (1988).
4. Bodner, M. *et al. Cell* **55,** 505–518 (1988).
5. Ingraham, H. A. *et al. Cell* **55,** 519–529 (1988).
6. Ko, H.-S., Fast, P., McBride, W. & Staudt, L. M. *Cell* **55,** 135–144 (1988).
7. Sturm, R. A., Das, G. & Herr, W. *Genes Dev.* **2,** 1582–1599 (1988).
8. Scheidereit, C. *et al. Nature* **336,** 551–557 (1988).
9. Müller, M. M., Ruppert, S., Schaffner, W. & Matthias, P. *Nature* **336,** 544–551 (1988).
10. Clerc, R. G., Corcoran, L. M., LeBowitz, J. H., Baltimore, D. & Sharp, P. A. *Genes Dev.* **2,** 1570–1581 (1988).
11. He, X. *et al. Nature* **340,** 35–42 (1989).
12. Bürglin, T. R., Finney, M., Coulson, A. & Ruvkun, G. *Nature* **341,** 239–243 (1989).
13. Monuki, E. S., Weinmaster, G., Kuhn, R. & Lemke, G. *Neuron* **2,** 783–793 (1989).

14. Sturm, R. A., Das, G. & Herr, W. *Nature* **336**, 601–604 (1988).
15. Lenardo, M. J. *et al. Science* **243**, 544–546 (1989).
16. Schreiber, E., Matthias, P., Müller, M. M. & Schaffner, W. *EMBO J.* **7**, 4221–4229 (1988).
17. Herr, W. *et al. Genes Dev.* **2**, 1513–1516 (1988).
18. Otting, G. *et al. EMBO J.* **7**, 4305–4309 (1988).
19. Garcia-Blanco, M. A., Clerc, R. G. & Sharp, P. A. *Genes Dev.* **3**, 739–745 (1989).
20. Qian, Y. Q. *et al. Cell* **59**, 573–580 (1989).
21. Mitchell, P. J. & Tjian, R. *Science* **245**, 371–378 (1989).
22. Kozak, M. *Cell* **47**, 481–483 (1986).
23. Connolly, T. & Gilmore, R. *J. Cell Biol.* **103**, 2253–2261 (1986).
24. Perara, E., Rothman, R. E. & Lingappa, L. *Science* **232**, 348–352 (1986).
25. Siegel, V. & Walter, P. *EMBO J.* **7**, 1769–1775 (1988).
26. Connolly, T., Collins, P. & Gilmore, R. *J. Cell Biol.* **108**, 299–307 (1989).
27. Kozak, M. *J. Cell Biol.* **108**, 229–241 (1989).
28. Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J. & Rutter, W. J. *Biochemistry* **18**, 5294–5299 (1979).
29. Ruppert, S., Müller, G., Kwon, B. & Schütz, G. *EMBO J.* **7**, 2715–2722 (1988).
30. Okamoto, K. *et al. Cell* **60**, 461–472 (1990).

ACKNOWLEDGEMENTS. We thank C. Peiker and T. Schowalter for technical assistance, A. K. Hatzopoulos for the *oct-2* probe, A. Stoykova for the tissue blot, G. Dressler, A. K. Hatzopoulos, C. Lobe and A. Püschel for comments on the manuscript, R. Altschäffel for photographic work, and H. P. Geithe for oligonucleotide synthesis. N.S. is supported by the Behrens-Weise Foundation. This work was supported by the Max-Planck Society and by the Bundesministerium für Forschung und Technologie. S.R. and N.S. contributed equally to this work.

Peptide ligand-induced conformation and surface expression of the L^d class I MHC molecule

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NEWLY synthesized major histocompatibility complex (MHC) class I molecules in the endoplasmic reticulum are thought to bind peptides of foreign and endogenous antigens[1–4]. Several lines of evidence indicate that β-2 microglobulin (β_2m) and/or peptide ligand participate in the intracellular transport and surface expression of class I molecules, but the nature of their involvement is still unclear[5,6]. Here we present evidence that culturing non-mutant cells (fibroblast, thymoma or mastocytoma) with a peptide ligand specific for the L^d class I molecule of the mouse leads to a dramatic (fourfold) and specific induction of L^d surface expression. Surprisingly, this peptide ligand-induced expression of L^d does not result in an increased intracellular association of L^d with β_2m. These findings demonstrate that the previously reported decrease in surface expression of L^d results from its failure to be saturated with endogenous self-peptide ligands. This unique feature of L^d could also contribute to the fact that several virus-specific cytotoxic T cell responses have been found to be L^d-restricted.

Certain class I molecules such as human HLA-C[7–9] molecules and the L^d and D^k molecules of the mouse[10,11], are distinguished by their weak associations with β_2m, their slower intracellular transport and/or lower cell-surface expression. Of these class I molecules, the L^d molecule has been most extensively investigated[11–13]. To test whether β_2m is the limiting factor controlling the transport and expression of L^d molecules, we produced a cell line in which β_2m was overexpressed relative to L^d and found no significant increase in L^d transport or surface expression (data not shown). Because this suggested to us that β_2m was not the limiting factor, we next investigated whether peptide ligand controls L^d expression using peptides derived from the murine cytomegalovirus (MCMV) or the tum$^-$ P815 tumour variant. These peptides have been shown specifically to use L^d as the restricting element for cytotoxic T lymphocyte (CTL) recognition[14,15]. We cultured L^d-positive cell lines in medium with or without peptide as described[6]. L-L^d, an L-cell fibroblast line transfected with the L^d gene, was cultured for

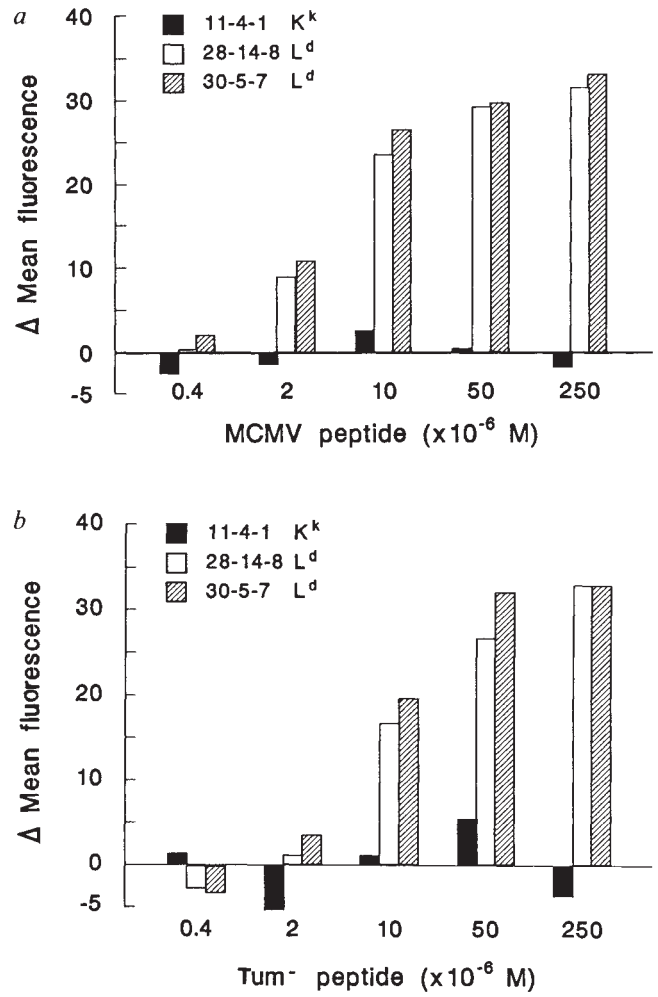

FIG. 1 Specific induction of L^d surface antigens on an L^d gene-transfected L-cell line (L-L^d). *a*, Dose response of L-L^d cells to MCMV peptide. The relevant amino-acid sequence of the MCMV peptide corresponds to residues 168–176 (Tyr-Pro-His-Phe-Met-Pro-Thr-Asn-Leu) of the murine cytomegalovirus (MCMV) immediate-early protein pp89 (ref. 14). *b*, Dose response of L-L^d cells to tum$^-$ peptide. Amino-acid sequence of the tum$^-$ peptide corresponds to residues 12–24 (Ile-Ser-Thr-Gln-Asn-His-Arg-Ala-Leu-Asp-Leu-Val-Ala) of the mutant protein P91A$^-$ (exon 4) from the tum$^-$ P815 variant[15]. A control D^b-restricted peptide, influenza NP 365–380 (ref. 6) did not induce expression of L^d (data not shown). L-L^d cells were exposed to various concentrations of peptide for 15 h and then stained with monoclonal antibody 11-4-1, 28-14-8, or 30-5-7 to detect K^k, L^d (α3), or L^d (α1/α2) antigens respectively. The results were obtained with logarithmic amplification of fluorescence intensity expressed in channels, where 4 logs span 256 channels (64 channels per log). Our FACS machine has been calibrated such that the relative increase is equal to 10^e, where $e = \Delta$ mean fluorescence (channels)/64 (channels/decade). According to this formula, an increase in fluorescence intensity of 40 channels represents a 4.2-fold increase in expression and 30 channels represents a 3-fold increase in expression.

METHODS. Peptides MCMV pp89 (168–176) and tum$^-$, P91A$^-$ (12–24), were made using an applied Biosystems Model 430A peptide synthesizer and standard t-Boc chemistry. 3.3×10^5 cells per ml were cultured with either medium (DMEM/10% FCS) alone or with various concentrations of peptide for 15 h at 37 °C, 6.5% CO_2. After spinning, cells were resuspended in Hanks balanced salt solution containing 2% BSA and 0.2% sodium azide and analysed by indirect immunofluorescence. A saturating concentration of monoclonal antibody 11-4-1, 28-14-8, or 30-5-7 was used to detect K^k, L^d (α3) and L^d (α1/α2) antigens respectively[24,25]. As a developing reagent, we used a saturating concentration of fluorescein-conjugated F(ab')2 fragment of goat anti-mouse IgG, Fc-specific (Organon Teknika-Cappel). Cells were analysed on a fluorescence-activated cell sorter (FACS IV, Becton Dickinson) and fluorescence histograms generated with logarithmic amplification of fluorescence emitted from single viable cells. Background staining did not vary significantly when treated with peptide or negative control antibody.