Mapping of the Transcriptional Repression Domain of the Lymphoid-specific Transcription Factor Oct-2A*

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The lymphoid-specific transcription factor Oct-2a is implicated in B cell-specific transcriptional activity via the octamer motif. Structure/function analysis of various Oct-2a effector regions in the context of the GAL4 DNA-binding domain revealed that Oct-2a contains two functionally different activation domains at the N and the C termini. The transcriptional activity of both domains is strongly potentiated by interactions with distinct B cell-specific coactivators. Recently, we have identified a repression domain located within the N-terminal region of Oct-2a (amino acids 2–99). When this domain was transferred to a potent activator, transcription was strongly inhibited. In this study we present a deletion analysis of the N-terminal region of Oct-2a to determine the minimal repression domain. We identified a stretch of 23 amino acids, rich in serine and threonine residues, which was responsible for most of the repression activity. We show that repression is strongly dependent on the type of enhancer present in the reporter plasmid as well as on the cell line tested. The possibility that Oct-2a can act as an activator and/or a repressor may have important consequences for the function of Oct-2a in B cell differentiation and other developmental processes.

The B cell-restricted expression of immunoglobulin (Ig) genes is dependent on promoter and enhancer elements that confer B cell-specificity. The octamer motif ATGCAAAAT or its inverse complement is a pivotal regulatory element for transcription of all Ig genes. It is conserved in all Ig promoters and most of the Ig enhancers (1).

Recently, a variety of transcription factors binding to the octamer site have been cloned (2), including the ubiquitously expressed Oct-1 protein (3) and the lymphoid-specific factor Oct-2a, was generated with the oligonucleotide primers 5'-TGATCTTTGTACTG-3' and 5'-AGGATCCGTTCACTCCAGCATGGG-3'. The N5 fragment encoding the amino acids 65–99, and its inverse complement is a pivotal regulatory element for transcription of all Ig genes. It is conserved in all Ig promoters and most of the Ig enhancers (1).

For a long time research has focused mainly on activator and coactivator proteins (15–17), but recently it became clear that repressors and corepressors also play an important role in regulation of gene expression (18–20). Repressors can act by passive repression strategies such as competition for DNA-binding sites, quenching, squelching, or the establishment of a repressive chromatin structure around the target promoter. Active repression mechanisms are also involved and imply a direct or indirect (using a corepressor) interaction with the transcription machinery, thus interfering with preinitiation complex assembly (18). Interestingly, several of the transcription factors investigated function as both activators and repressors depending on the target promoter and the cellular context (21). This could also be the case for Oct-2a, which belongs to a group of transcription factors with a complex modular structure consisting of separable domains for activation and repression (12), similar to Krüppel (22), Even-skipped (22), Egr-1 (23), and YY1 (24).

In this study we describe a deletion analysis of the N-terminal domain of Oct-2a (N1 region) in order to define the region involved in transcriptional repression. Different segments of the N1 region were fused to the potent activator protein GAL4-C and tested in transient transfections of B cells and non-lymphoid cells together with luciferase-based reporter plasmids containing different enhancers. We show that the major repressor activity mapped to a region of 23 amino acids with a high abundance of serine and threonine residues (30.4%). Furthermore, we demonstrate that transcriptional repression displayed cell type-specific characteristics, which were determined by the enhancer present in the reporter plasmid.

Materials and Methods

Construction of Plasmids—The expression vectors pGAL4, pGAL4-C-C, pN1-GAL4-C-C, and pN2-GAL4-C-C were constructed as described previously (12). pGAL4 encodes the DNA-binding domain of the yeast transcription factor GAL4 (residues 1–147). The expression vector pGAL4-C-C encodes a fusion protein containing GAL4 (residues 1–147) linked to a duplicated Oct-2a C terminus (residues 463–479). The expression vectors pN1-GAL4-C-C and pN2-GAL4-C-C were made by linking the N-terminal fragments N1, encoding amino acids 2–99, and N2, encoding residues 163–207, of Oct-2a to pGAL4-C-C. The chimeric constructs pN3-GAL4-C-C, pN4-GAL4-C-C, and pN5-GAL4-C-C were generated by introduction of the Asp718-BamHI-deleted polymerase chain reaction fragments N3, N4, and N5 into the respective sites of pGAL4-C-C. The N3 fragment encodes the amino acids 2–64 of Oct-2a and was amplified with the primers 5′-TACCAGGATCCGTTACCTCCAGCATGGG-3′ and 5′-GTCCTTGGTACCCCTTGGTACTG-3′. The N4 region, encoding residues 42–99 of Oct-2a, was generated with the oligonucleotide primers 5′-TAGAGGATCCGTTACCTCCAGCATGGG-3′ and 5′-CATCTTGGTACCCCTTGGTACTG-3′. The N5 fragment, encoding Oct-2a residues 65–99,
COS-7 cells were transiently transfected with 3 \( \mu \)g of the various expression vectors. The binding reaction was carried out with 2.5 \( \mu \)g of COS-7 nuclear cell extract and 40 fmol of a \([\gamma^{32}\text{P}ATP}\) end-labeled oligonucleotide probe containing one GAL4-binding site. Protein-DNA complexes and free probe were separated by electrophoresis through a native 4% polyacrylamide gel (80:1) at room temperature. Lane 1, free probe; lanes 2–8, expressed GAL4 fusion protein as indicated above each lane; lane 9, extract of mock-transfected cells.

Assay (EMSA)—Nuclear extracts from transfected COS-7 cells were prepared by a small scale procedure (27). EMSA reactions (28, 29) were set up in 20 \( \mu \)l with 2.5 \( \mu \)g of nuclear extract, 40 fmol of an end-labeled probe containing a single Gal4-binding site, 0.5 \( \mu \)g of denatured salmon sperm DNA (Pharmacia), and 1 \( \mu \)g of poly(dI-dC) (Pharmacia) in a buffer containing 20 mM HEPES, pH 7.9, 50 mM KCl, 5 mM MgCl\(_2\), 10 \( \mu \)M ZnCl\(_2\), 6% glycerol, and 200 \( \mu \)g/ml bovine serum albumin. Binding reactions were incubated for 15 min at room temperature and resolved at 15 V/cm for 2 h on a native pre-run 4% polyacrylamide gel (80:1) containing 0.5 \( \times \) TBE and 1% glycerol. The gel was fixed for 30 min in 20% (v/v) methanol and 10% (v/v) acetic acid, dried, and autoradiographed.

Luciferase Assays—Luciferase assays were performed essentially as described previously (30, 31) using a Berthold MicroLumat LB96P machine. Whole cell extracts were prepared by three cycles of freeze-thaw lysis in 0.1 M potassium phosphate, pH 7.8, and 1 mM dithiothreitol. The luciferase activity was measured in a total volume of 200 \( \mu \)l containing 100 \( \mu \)g of the protein extract, 11.25 \( \mu \)l MgSO\(_4\), 18.75 \( \mu \)l glycine, 2.0 \( \mu \)l ATP (Boehringer Mannheim), and 0.15 \( \mu \)l of the substrate d-luciferin (Sigma), pH 7.8. Light output was measured for 15 s.

**RESULTS AND DISCUSSION**

In our previous experiments, we have shown that Oct-2a is a complex transcription factor consisting of separable modules for activation and repression. The repression domain was localized to the N-terminal 99 amino acids of Oct-2a, the N1 region (12).

To further delineate the domain important for repression, we have performed a deletion analysis of the N1 region. Various expression vectors were generated containing different regions of the N1 segment, comprising the repression domain, fused to a potent activator protein. The regions N1 (amino acids 2–99), N2 (amino acids 163–207), N3 (amino acids 2–64), N4 (residues 42–99), and N5 (residues 65–99) of Oct-2a were linked to the Gal4 DNA-binding domain (aa 1–147). GAL4-C-C contains the duplication C-terminal of Oct-2a (aa 363–479). The regions N1 (aa 2–99), N2 (aa 163–207), N3 (aa 2–64), N4 (aa 42–99), and N5 (aa 65–99) of Oct-2a are fused to the potent activator protein GAL4-C-C to test their repression activity. In A and B, the structural representation of the domains is drawn to scale.

was amplified using the oligonucleotide primers 5'-GGTAGGATCCGCTGAAGACCCAGTG-3' and 5'-CATCTTGGTACCTAGCTGGCGCCGTC-3'. The constructs were verified by restriction analysis and DNA sequencing. The luciferase reporter plasmids p2GlucE and p2GlucBG5 have been described previously (12). The target vector p2GlucBG5 contains two Gal4-binding sites in the promoter region 13 base pairs upstream of the TATA box in the context of a minimal rabbit \( \beta \)-globin promoter and a simian virus 40 (SV40) enhancer. 2.85 kilobase pairs downstream of the transcription start site. The luciferase reporter plasmid p2GlucBG5 has the same structure as p2GlucE but contains, instead of the SV40 enhancer, a synthetic enhancer consisting of five Gal4-binding sites.

**Preparation of Nuclear Extracts and Electrophoretic Mobility Shift**
Repression by Oct-2a

A. HeLa and Namalwa cells were cotransfected with 6 μg of luciferase reporter plasmid p2GlucE and 4 μg of various expression vectors containing potential repression domains linked to the potent activator GAL4-C-C. B, the luciferase reporter plasmid p2GlucBG5 was used for cotransfections into HeLa and Namalwa cells together with the expression vectors used in A. For A and B, transfections and luciferase assays were performed as described under "Materials and Methods". The activity of GAL4 (aa 1–147) on the target vectors p2GlucE and p2GlucBG5 was arbitrarily set to 1 in both cell lines tested. The activation potential of the constructs was expressed relative to GAL4 (aa 1–147). Standard deviations of at least five independent experiments are shown as error bars.

Repression of the reporter plasmid p2GlucBG5 by N4-GAL4-C-C, and no repression was seen with N5-GAL4-C-C and the control N2-GAL4-C-C in agreement with the results presented in Fig. 3A. Interestingly, transcriptional repression with the reporter plasmid p2GlucBG5 was observed in all of the cell lines tested. Again N4-GAL4-C-C repressed transcription to the same extent as N1-GAL4-C-C, whereas N5-GAL4-C-C did not inhibit transcription in HeLa cells and had only a minor effect in Namalwa cells.

As shown in Fig. 3, the repression domain of Oct-2a can be mapped to residues 42–64. The relative position of the various domains tested (N1, N2, N3, N4, and N5) within the Oct-2a N terminus and the minimal repression domain (dark box) are outlined in Fig. 4.

As mentioned above, region N4 represses transcription to the same extent as the extended N1 region. However, the N3 domain is slightly less efficient than N4 in inhibiting transcription despite containing the minimal repression domain. This may be due to a slight destabilization of the protein structure of the N3 domain, since a high probability for a β sheet was predicted for exactly the C-terminal end of N3 (program PredictProtein), containing the inhibitory domain. This region could be stabilized by the additional C-terminal residues present in the N4 domain. These residues could favor the correct folding of the inhibitory domain giving rise to maximal transcriptional repression.

Repression of the reporter plasmid p2GlucBG5 by N4-GAL4-C-C was observed in both HeLa and Namalwa cells. In contrast, repression of the target vector p2GlucE was only seen in Namalwa cells and not in HeLa cells. This is consistent with our previous data (12) and underlines that repression by Oct-2a follows cell type-specific characteristics that are strongly determined by the enhancer present on the reporter plasmid. Therefore, we conclude that repression by N4-GAL4-C-C is impaired by a HeLa cell-specific factor(s) interacting with the SV40 enhancer. Such a factor(s) could interfere with repression by masking the inhibitory domain of Oct-2a. This working model is in agreement with the finding that the N terminus of Oct-2a can exert its negative effect when linked to other Oct-2a activation domains only if HeLa cell-specific factors binding to the SV40 enhancer are not present (Namalwa cells) or are not able to bind (synthetic Gal4 enhancer) (12).

Region N4, containing the minimal repression domain between residues 42–64, is a transferable inhibitory domain that works independently of its cognate DNA-binding POU domain. N4 linked to the activator GAL4-C-C efficiently inhibits transcription and can therefore be classified as an active transcriptional repressor (19, 20, 32). This class of repression domains is
implicated in contacting components of the basal transcription apparatus, thus interfering with the formation of a transcription-competent initiation complex. This was recently demonstrated for Dr1 (33) and the Drosophila proteins Krüppel (34) and Even-skipped (35). In the context of the native Oct-2a protein, repression could follow a combination of different mechanisms including competition for DNA-binding sites and quenching, both requiring the presence of the homologous DNA-binding POU domain, or interaction with basal transcription factors. This is not unexpected since different mechanisms of repression, depending on the promoter architecture and the type and amount of distinct factors present in the cellular context, are used by repressor proteins such as Krüppel (22, 34, 36) and Even-skipped (35, 37).

The repression domain of Oct-2a, which mapped to amino acids 42–64, is rich in serine and threonine residues (30.4%). Protein data base searches with this region did not reveal a significant homology to other known transcriptional repressors, and no particular structural motifs were found. Therefore, this region could represent a novel type of repression domain. Experiments to identify possible targets of the Oct-2a inhibitory domain using a yeast two-hybrid system are currently in progress in our laboratory.

Recently, negative regulatory domains have also been identified within other isoforms of Oct-2 (13, 14). These domains are neither related to each other nor to the repression domain mapped in this study. One of these regions was assigned to an N-terminal domain common to both the neuronally expressed mouse isoforms Oct 2.4 and Oct 2.5 (13). This region specifically inhibited activation of a herpes simplex virus immediate-early promoter containing the octamer/TAATGARAT motif and was mapped in this study. One of these regions was assigned to an expression-competent initiation complex. This was recently demonstrated for Dr1 (33) and the

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