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International Centre for Genetic Engineering and Biotechnology
United Nations Industrial Development Organization

20761

Collaborative Research Programme

TERMINAL EVALUATION REPORT

UNIDO contract #

91/058

ICGEB ref. #: CRP/

HUN90-06

Project initiation:

1991

Project termination:

1994

Principal investigator
Prof. László Orosz

International Centre for Genetic Engineering and Biotechnology
United Nations Industrial Development Organization

Collaborative Research Programme

TERMINAL EVALUATION REPORT

Part 1

Title of Project

Structural studies on sequence specific DNA-protein interaction

Keywords: Transcription regulation, DNA binding motif, helix-turn-helix, 16-3 and 434 repressor, Rhizobium phage, DNA-looping

UNIDO contract # 91/058 **ICGEB ref. #:** CRP/ HUN 90-06

Project initiation: 1991 **Project termination:** 1994

Principal Investigator's name: LASZLO OROSZ

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Abstract:

It was discovered that a regulatory protein (here the 16-3 repressor) can accomodate and bind to at DNA segments of different lengths and twists (here 16-3 operators) with high specificity. The significance of these studies and discovery are manyfold:

(i) this (first observed) example opens new untouched field for investigating the protein/DNA sequence specific bindings, namely the role of conformations, and their structural preconditions.

(ii) questions emerged of general importance as:
- how do the conformations of protein and/or DNA accomodate the spacial and rotational differences in sequence-specific binding.
- how does the overall specificity of operator/repressor interaction assemble from the contributing elements (DNA-protein atomic contacts, DNA looping, binding, repeated binding sites, cooperativity)

OBJECTIVES/METHODOLOGY
(proposed at the time of the submission of the research proposal)

Objectives

Background: Our previous work on the repressor/operator system of phage 16-3 of Rhizobium meliloti suggested: (i) limited number of specificity classes (for repressor/operator binding) may exist in nature; (ii) unrelated DNA binding proteins can indeed have identical or similar binding pattern; (iii) one repressor molecule might interact very specificly with significantly different operators

Objectives:

1. The general goal of our work was to gain insight into the molecular basis of DNA/protein interactions.

2. Our previons work demonstrated that DNA binding specificity of repressor of phage 16-3 was related to DNA bindig specificity of unrelated E.coli phage 434. Setting out from this result we proposed (i) a systematic study on the domain structure/function (ii) comparative investiqations on DNA binding with chemically synthetized peptides based on 434 and 16-3 repressors, both having helix-turn-helix motifs.

3. DNA sequences of two operator regions of 16-3, O_L and O_R suggested that O_L and O_R might significantly be different. O_R was identical to the 434 operators, while the putative O_L sequences were shorter by two base pairs. We suggested detailed analysis of the operator regions, since if the O_R and O_L operators really differed from each other as believed, this would raise very significant general questions concerning the geometry of the interface of the specific protein/DNA complex (i.e. repressor/operator). Namely: how the same protein (repressor) could tolerate differences like 2 base pairs in length and 72° in rotation when binding (with extremely high specificity) to DNA (operator).

Methodology
We proposed:
- In vivo functional studies with cloned repressor and operator alleles in Rhizobium meliloti. The operator/repressor interactions were planned to monitor by measuring the expression of appropriate reporter gene systems.
- In vitro binding studies with purified repressors and operators: band shift assays; repressor/operator "footprintings".
- Comparison of the in vivo and in vitro approaches
- In vitro investigations with synthetic DNA binding peptides and DNA oligos.
- Computer graphics, conformational energy calculations (in order to estimate the stability of the interactions).

RESULTS 1.
(compare against the set objectives)

On proposals vs. results (in general)

As quite usual, perhaps rather rule than exception with basic research, the actual work develops differently from that put down in the original proposal. Here in our case the in vivo approaches (with cloned operators/repressor genes; with a rich set of experimental lineups; with induced and site directed mutants, with reporter genes in homogeneous system - in Rhizobium meliloti etc.) progressed faster, produced extra, unexpected, very significant results in the "main line" (see "DNA looping") as well as along the allied "tributaries" (see "att site").
The in vitro analyses had been hampered for long by the difficulties of the isolation of conformationally active, solubile 16-3 repressor. The binding conditions proved very extreme, which also consumed lots of time for establishing "band shifts" and "footprintings".
The concept of the "minimum system" envisaged would have based entirely on sythetic components, DNA binding peptide motifs and DNA oligos for operators, proved premature. The experimental "arsenal" is still not ripe enough for establishing "minimum binding systems" for the 20 amino acid long helix-turn-helix motifs. However at "semi minimum" scale (i.e. when a peptide of 70-80 aminoacids folds into the DNA binding structural domain with helix-turn-helix recognition motif included) a significant progress, we can report about.

Results

1./ On the domain structure of the 16-3 repressor:

1.1. The DNA binding domain is highly autonomous and biologically active in vivo and in vitro

Our results prove that the Rhizobium phage 16-3 repressor folds into domains. One, the operator binding domain can autonomously regulate on the 16-3 operator O_R. The results of three experiments have supported this conclusion.

(i) Repressors whose carboxyl-terminal regions had been deleted repressed O_R in vivo. The most severely truncated active variant lost 60% of the 197 amino acid long repressor. This repressor, R1-77, retains only the first 77 amino acids of the intact protein. (For these studies, we constructed an appropriate reporter plasmid system, in which the effect of operator and repressor alleles, their combinations, can be characterized quantitatively. It is worth mentioning that these repressors were probably not overexpressed, since the truncated repressor genes were cloned in low-copy-number plasmids).

(ii) A fusion protein R1-77-ß-gal binds to the O_R operator in vitro. (We constructed a fusion gene, which in E. coli directed the synthesis of a protein having the R1-77 repressor at its amino terminal region fused to ß-galactosidase residues 8-1021. Two alleles of this fusion gene were constructed: one with the wild type R1-77 region and one with the temperature sensitive mutant allele of the same region. The DNA binding abilities of the hybrid proteins were investigated by gel retardation assays, using wildtype and mutant operator DNAs).

(iii) Mutations in the amino-terminal domain affected the ability of the repressor to block the reporter gene expression from P_R, while a mutation mapping outside this region, in the carboxyl terminal half of the protein, did not. There were two sources of mutant aleles: from temperature sensitive - i.e. missense - mutants, and from in vitro made mutations - which were transferred to the 16-3 chromosome, and their phenotypic effects tested in the resulting phage mutant - "reversed genetics". The technique to transfer an allele - made in vitro in E. coli - to a Rhizobium specific phage (16-3), and to regain the mutant specimen, was developed in our laboratoy.

1.2. The "carboxyl-end domain" and asymmetry in charge between regions.

The 16-3 repressor was isolated and purified to homogeneity (see more in section 1.3.).
Two natural variants of 16-3 repressor were identified in 50-50% by N terminal sequencing - the shorter was 189 amino acids long, the longer protruded by 8 amino acids at the N terminus. Both variants corresponded to the cognate AUG codons of the ORF of the C repressor gene of 16-3. The significance of this heterogeneity is not known.
At certain conditions the repressor broke specifically which made possible to isolate the complete C-terminal domain. The exact site (i.e. peptide bond) of this fracture was idetified by N terminal microsequencing. The complete repressor is basic (pI 9.2.) and asymmetric in charge: while the C-terminal domain is acidic (pI 5.3) the N-terminal DNA binding domain (R1-77) is extremely basic (pI 11.4). The active repressor was dimer, but higher degree oligomers have also been detected.

1.3. Overexpression of the 16-3 repressor.

E. coli expression system was developed for this purpose, having used tac promoter and pKS plasmid backbone. Half of the crude protein was active 16-3 repressor according to the criteria of band shift assays and footprintings with/on O_R operator DNA.
It is worth mentioning that prior to the above described expression system we developed an other version (based on λ pL and λ CIts control) which produced even more repressor, however with small fraction having been solubile and active in DNA binding tests.

2./ <u>One repressor for different operators: O_L and O_R</u>

2.1. Mutant repressors distinguish between O_L and O_R

The loss of the carboxyl terminal region as well as mutations in certain segment of the repressor still allows binding at one of the operators at O_R. However these mutant repressors are unable to maintain lysogeny, are unable to control over the O_L operator. Hence, we concluded that the carboxyl terminal region plays an indispensable role in other significant processes required for gene control (i.e. for lysogeny).

2.2. Operator O_L is 2 base pairs shorter than O_R.

Mutations were induced in vivo (utilizing immunity insensitive phenotype) and in vitro (site directed) in the operators; mutant operator alleles were introduced into the tester system. Plasmid systems (integrative/compatible to) for Rhizobium system were developed: containing LacZ for reporter gene, fused to multicloning site, where 16-3 operator-promoter units (allelic variants) were inserted. The repressor was provided from trans from an integrated proghage. The final and apparent outcome of these in vivo studies are:

- The O_R operator is 14 bp long and palindromic: 5'ACAA 6bp TTGT3'
- The O_L operator is shorter, 12 bp long with the same palindromic structure: 5'ACAA 4bp TTGT3'
- There are two O_L operators, O_{L1} and O_{L2}, separated by 1.6 helical turns, hence they occupy the opposite surfaces on DNA. Both O_{L1} and O_{L2} are biologically active, since mutations at either lead to derepression of LacZ.

3./ <u>High specificity of operator/repressor binding and very strong cooperativity between operator bound repressors.</u>

Our tester system made possible to measure and calculate the tightness of binding between operators and the repressors. Dissotiation constants (Kd) were in the range of 10^{-10} M for both O_L and O_R operator/repressor complexes.

However if an O_R-O_L couple was in control the Kd value dropped 20 fold, in the range of 10^{-12} M, indicating very strong repressor/operator binding and cooperativity. This cooperativity controls (blocks) promoter activities at both directions, left and rigthward.

4./ <u>On the nature of cooperativity between operators: DNA looping involved?</u>

Plasmids carrying operator O_R-O_L couples were constructed in order to test phase and distance sensitivity of OR-OL cooperativity. The operator sites were separeted by 11.2, 11.6, 12, 12.4 helical turns, and up to a few kb in B DNA.
To date no phase sensitivity has been detected, an indication for flexible structures (in the operator/repressor/DNA-loop complex), which tolerated the steric differences introduced by the variety of helical twits of the DNA streches separated O_L and O_R operators.

5./ <u>Methods developed and adapted (for investigating 16-3 repressor/operator system).</u>

- Promoter probe plasmid systems (utilizing LacZ for reporter gene) for Rhizobium meliloti, proper for accurate monitoring and quantitative characterization of repressor/operator interactions.
- Plasmid system for testing DNA looping

- Expression system for overproducing 16-3 repressor, purification of repressor.
- Band shift assays with 16-3 repressor and operators
- DNaze footprinting on 16-3 repressor/O_R operator complex.
- Site directed mutagenesis for inducing mutant operators and repressors.
- Peptide design, dimerization by S-S bridge.
- N-terminal microsequencing of peptides
- Transverse gradient gel ELFO, for detecting DNA bending

6./ Studies with synthetic peptides.

We designed various peptides with helix-turn-helix motifs of 16-3 and of 434 repressors (the two repressors have related DNA binding specificity). Folding and DNA binding properties were investigated in Trieste by S. Pongor (in ICGEB Centre).

7./ Significant "side" result: the attachment site of 16-3 is a part of a pro tRNA gene.

We developed an integrative version of the plasmid sets constructed for monitoring the operator/repressor interactions (see at section 5.). We utilized the 16-3/R. meliloti integrative recombination: the attP and attB sites, and the int/xis genes/functions. The raticnale was twofold: (i) the reporter operon could be inserted in the R. meliloti chromosome, hence can be studied in one copy; (ii) otherwise incompatible plasmid pairs could be used (one integrated in the chromosome, the other maintained in the cytoplasm). During this methodical development we characterized the att/int system of 16-3. Unexpected and significant results have arisen: (i) the attB site (51 bp) overlapped the 3' end of a pro-tRA gene of the R. meliloti chomosome; (ii) related integrative system was discovered in the unrelated Actynomycetes.

8./ The most important conclusions:

Questions of general significance, concerning the steric geometry for Protein/DNA sequence specific interactions have arisen:

(i) how and what structures of the repressor protein can accomodate to spacially and rotationally different DNA streches;

(ii) how can two operator bound repressor proteins communicate with each other when occupy either opposite surfaces on, or very distant loci along DNA.

(iii) How much the contribution of the atomic contacts (between operator and binding motif), of the DNA conformation, of the cooperativity (DNA looping), of the doubled binding sites, to the overall specificity of sequence specific binding.

Work plan	Work plan and time schedule (originally envisaged)

I repeat here the relevant section of our ICGEB grant proposal from 1991:

"We would compare the in vitro results with the in vivo investigations: (i) the O_L and O_R operators (of phage 16-3) as well as the repressor gene will be saturated with mutations and the effect of each mutation will be tested in vivo in reporter gene system; (ii) the "footprints" of intact (16-3 and 434 phage's) repressors will be compared to the footprints done by the helix-turn-helix motifs (of 16-3 and 434 repressors); (iii) the strength of binding will be compared in different combinations of synthetised helix-turn-helix motifs and operator oligonucleotides. Consistencies between results (i) vs. (ii) and (i) vs. (iii) will be tested: (i) vs. (ii) should define the length and the repressor covered surface of the operator(s) DNA; (i) vs. (iii) may define the biologicaly important molecular (or even atomic) contacts between DNA bases of the operator and amino acid side chains in the repressor.

Design: Computer graphics and molecular energy calculations (Silicon Graphics workstation using softwares AMBER and CHARMM), in cooperation with ICGEB, Trieste.

Synthesis: Solid phase peptide synthesis using an Applied Biosystems peptide synthesizer. Oligonucleotide synthesis will be carried out on a Pharmacia automated oligonucleotide synthesizer. Purification of the peptides and oligonucleotides will be carried out by reverse phase HPLC.

Structural studies by CD, NMR, and, if possible, X-ray diffraction will be carried out in cooperation with other groups in Hungary and at ICGEB, Trieste.

Biological experiments will be carried out by standard methods (recombinant DNA technology, phage and bacterium genetics, enzyme assays, DNA band shift etc.)"

Time schedule

For 1991

a.) Isolation of operator mutants in vivo and in vitro (by PCR aided site directed mutagenezis).
b.) Constructing overproducing plasmids for the 16-3 repressor and for the R1-77 domain.
c.) Constructing tester/reporter operon systems of compatible in R. meliloti.
d.) Sythesis of the helix-turn-helix peptides of 16-3 and 434 repressors with appropriate spacers designed.

For 1992

a.) band shift and footprinting assays with 16-3 and 434 repressors.
b.) characterising the operator (alleles)/repressor(alleles) control over 16-3 O_LP_L and O_RP_R directed transcription
c.) in vitro assays with synthetic DNA binding motifs: folding and DNA binding capacity

For 1993-94

Starting from the results provided by the 1991-92 years further progress proposed toward defining the "structure" of specificity of repressor/operator binding.

Work plan and time schedule (actual) 1.

Project landmarks, duration of individual tasks (use bar charts), evaluation criteria (publications, patents, services, training)

1./ The most significant and novel result of these experiments was the identification of the 16-3 O_L operator, namely that this native operator was shorter than the other cognate operators specific for binding 16-3 and 434 repressors. The difference in the length (i.e. in base pairs) introduces very significant, 36° rotational difference by each base pair.

The 16-3 repressor with its cognate operators, the 14 bp long O_R and the 12bp long O_L raised a steric problem of general importance: how the conformation of protein and DNA can accomodate to spacial and rotational differences in sequence specific interactions.

The duplicated O_L operators, O_{L1} and O_{L2}, ask the question: how can two operator bound repressor proteins communicate with each other when occupy opposite surfaces on DNA.

The lack of phase sensitivity of O_L-O_R cooperation asks backward: what protein and DNA structures ensure this flexibility.

2./ Very significant infrastructural development is the coherent and versatile experimental system constructed Rhizobium compatible:

- The plasmid sets with LacZ reporter gene which allows very accurate quantitative characterizations; which provides room for testing (at wish) combinations of operator alleles vs repressor alleles; which gives opportunity to test the effect of gene dosage (i.e. dominance/recessivity, negative and inter-allelic complementation for repressor alleles, in vivo binding test for operators); which provides sensitive method for assaying DNA looping.
- The method developed for transfering in vitro engineered repressor and operator alleles (back) to the chromosome, thus providing (in vivo) tool for testing the phenotypic effect of these alleles (clear example of inverted genetics).

3./ Also novel is the coherent system developed for in vitro binding studies (repressor overproducing strains, repressor purification, parameters for band shifts/for footprintings, for DNA looping tests, novel gel ELFO method for monitoring DNA bending).

Time schedule

1991

a.) Isolation of operator mutants in vivo and in vitro (by PCR aided site directed mutagenezis).
b.) Constructing overproducing plasmids for the 16-3 repressor and for the R1-77 domain.
c.) Constructing tester/reporter operon systems of compatible in R. meliloti.
d.) Sythesis of the helix-turn-helix peptides of 16-3 and 434 repressors with appropriate spacers designed.
e.) Characterizing the N-terminal, R1-77 domain of 16-3 repressor.
f.) Developing method for transfering in vitro made mutations to the chromosome.

Work plan and time schedule
(actual) 2.

Project landmarks, duration of individual tasks (use bar charts), evaluation criteria (publications, patents, services, training)

1992

a.) Attempts to isolate active 16-3 repressor.
b.) Attempts to clarify band shift conditions.
c.) Constructing integrative plasmid vectors utilizing 16-3/R.meliloti att/int/xis system.
d.) Characterizing the $attB_{16-3}$ region of R.meliloti.
e.) Identification of O_L^C mutants.

1993

a.) Constructing new plasmid versions (with tac promoter) to overproduce 16-3 repressor.
b.) Isolation of large quantity of pure 16-3 repressor.
c.) Developing band shift assays with O_R.
d.) Constructing plasmids for assaying DNA looping.
e.) Measuring Kd values for 16-3 repressor/O_L and O_R complexes; for O_L-O_R cooperativity (DNA looping).
f.) Studies (folding, dimerization) with large synthetic repressor peptides.

1994

a.) DNase footprintings on 16-3 repressor/O_R complex.
b.) Developing band shifts for O_L operators.
c.) Developing band shift test for detecting O_L-O_R cooperativity.
d.) Phase sensitivity assays of O_L-O_R cooperativity.
e.) Characterizing large sets of O_R^C and O_L^C mutations.
f.) Characterizing the C-domain of the represor.
g.) Optimizing footprinting assay for O_L operators.

Progress: summary comparison of expected (proposed) vs. actual
(repeated from page 3)

As quite usual, perhaps rather rule than exception with basic research, the actual work develops differently from that put down in the original proposal. Here in our case the in vivo approaches (with cloned operators/repressor genes; with a rich set of experimental lineups; with induced and site directed mutants, with reporter genes in homogeneous system - in Rhizobium meliloti etc.) progressed faster, produced extra, unexpected, very significant results in the "main line" (see "DNA looping") as well as along the allied "tributaries" (see "att site").
The in vitro analyses had been hampered for long by the difficulties of the isolation of conformationally active, solubile 16-3 repressor. The binding conditions proved very extreme, which also consumed lots of time for establishing "band shifts" and "footprintings".
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Publications

see page 6.

NETWORKING

Prof. Laszlo Orosz, principal investigator (genetic analysis, molecular biology)
ABC, Institute for Molecular Genetics, Godollo

Prof. Sandor Pongor, (protein chemistry, modelling) ICGEB, Trieste

Dr. Andras Patthy (sequencing and synthesis specialist)
ABC, Institute for Biochemistry and Protein Research, Godollo

Dr. Zsuzsanna Buzas (synthesis and ELFO specialist)
ABC, Institute for Biochemistry and Protein Research, Godollo

Prof. Andreas Chrambach (leading ELFO theoretician) NIH, NICHD, Bethesda

Prof. Sankar Adhya (leading theoretician of genetic regulation) NIH, NCI, Bethesda

Dr. Mark Garner (leading expert in the field of DNA/protein interaction) NIH, NICHD, Bethesda

PUBLICATIONS

1991 Dellmess, G., Mariecs, F., Papp, P., Gasmer, M. and Orosz, L. The isolated N-terminal DNA binding domain of the C repressor of bacteriophage 16-3 is functional in DNA binding in vivo and vitro. Mol. Gen. Genet. 227:106-113.

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FINANCIAL REPORT
1991-94

Project: GE/GLO 89/001 ICGEB

"Structural Studies on Sequence Specific DNA-protein Interaction"
UNIDO Contract No. 91/058
Ref. HUN 90 -06

Income (till 1994.06.23): USD 22000 = 1 728 000
Expenditure (till 1994.06.23):

Enzymes (restriction -,DNA polymerase, etc.)	321 000 HUF
Oligos	251 000 HUF
Izotopes (P^{32},S^{35})	135 000 HUF
Other fine chemicals	591 000 HUF
Glassware, small lab. items	153 000 HUF
Service (library, xerox, workshop)	147 000 HUF
Accomodation for Prof.Andreas Chrambach(15days)1992	20 000 HUF
Travelling expenses for Prof. Andreas Chrambach (Washington/Budapest) 1992	117 000 HUF
Capital equipment (1994)	748 000 HUF
Total	2 483 000 HUF*

HUF = Hungarian Forint

Gödöllő, Jún 23. 1994.

.....
Chif financial officer
Nagy Józsefné

.....
Principal Investigator
Prof. László Orosz

*1 USD
at 1992 spring 77 HUF
at 1992 autumn 84 HUF
at 1993 91 HUF
average 84 HUF

Part 4

STATEMENT OF EXPENDITURES

To be filled by ICGEB		To be filled by the Affiliated Centre	
Budgets as per original proposal		**Summary of expenditures** *	
1) Capital equipment	US$	1) Capital equipment	US$ 8905
2) consumables	US$	2) consumables	US$ 17500
3) training	US$	3) training	US$ 1631
4) literature	US$	4) literature	US$ ---
5) miscellaneous	US$	5) miscellaneous	US$ 1964
TOTAL GRANT	**US$.....**	**TOTAL**	**US$ 30000**

Please itemize the following budget categories (if applicable)

Capital equipment

UVP Image analysis apparatus (completed with the compatible computer) - our ICGEB source (budget) covered 47 % of the total price (1 600 000 HUF). This equipment improved considerably the infrastructure of the lab and the quality of our daily work.

Training (provide names, duration of training, host laboratory)

We invited to ABC Inst. Mol. Genet (Gödöllő) Prof. Andreas Chrambach (from NIH, NICHD, Bethesda) for two weeks (in 1992) to teach us the high resolution advanced ELFO techniques. Prof. Andreas Chrambach is the leading theoretician of the field. The need for his invitation was mentioned in the original proposal.

Literature

L. Kiss
.....
Chif financial officer
Nagy Józsefné

.....
Principal Investigator
Prof. Orosz László

* Please do not send invoices, receipts etc.; these should be kept by the Affiliated Centre for future reference and sent to ICGEB upon request.