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A versatile and highly repressible *Escherichia coli* expression system based on invertible promoters: expression of a gene encoding a toxic product

(Phage λ integrase; toxic gene products; single-chain T-cell receptor; conditional runaway-replication; antisense promoter)

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SUMMARY

A very flexible and tightly regulatable expression system has been constructed. It uses the principle of invertible promoters [Podhajska et al., Gene 40 (1985) 163–168]. Here, we describe the construction of a plasmid that provides the integrase, which causes promoter inversion in a tightly regulated fashion, as well as modified plasmids carrying the invertible module. The way the integrase is provided on a separate plasmid closely mimicks expression of the integrase from a λ lysogen. Thus, the flexibility of the original system is considerably extended by making it strain-independent without compromising the tight regulation. We present the expression of a single-chain T-cell receptor fragment as an example of application, in order to illustrate the properties of this expression system.

INTRODUCTION

A large number of systems for production of more or less toxic proteins in *Escherichia coli* have been described: most widely used are naturally repressible promoters, e.g., the *lac* system. Most of these promoter/operator systems are very easily applied to a specific expression problem, but their dynamic range is limited (Lanzer and Bujard, 1988). To obtain higher levels of repressibility, several artificial systems have been described that use a natural repressible promoter but subject it to an additional level of control (Larsen et al., 1984; Podhajska et al., 1985; Tabor and Richardson, 1985; O'Connor and Timmis, 1987; Studier et al., 1990).

The basis of the system of Szybalski and co-workers (Podhajska et al., 1985; Hasan and Szybalski, 1987) is the following: The promoter, flanked by two λ attachment sites, is cloned in a direction opposite to the gene to be expressed and is inverted by inducing site-specific genetic recombination using the λ integrase. An opposite orientation of the promoter with respect to the gene to be expressed can thus be converted to a right orientation. Due to the presence of only the integrase and the attachment sites this process is inherently unidirectional. Hasan and Szybalski (1987) provided the integrase by making the expression strain lysogenic for λ *xis*⁻ *kil*⁻ *cI*857.

In trying to functionally produce the variable domains of a T-cell receptor (TCR) we were in need of a highly repressible system, since initial experiments suggested that the soluble, undegraded TCR is toxic. This system should furthermore be strain-independent and allow facile genetic manipulations in order to increase or decrease the expression level. The invertible promoter

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Abbreviations: Ap, ampicillin; bp, base pair(s); MCS, multiple cloning site; nt, nucleotide(s); oligo, oligodeoxyribonucleotide; *ori*, origin of DNA replication; PCR, polymerase chain reaction; sc, single chain; TCR, T-cell receptor; *tsp*, transcription start point(s); [], denotes plasmid-carrier state.

system seemed, apart from its limitation of needing a particular expression strain, the most suitable basis for developing such an expression system.

Here we describe a plasmid that allows any strain to invert the invertible promoter module, as if the strain were lysogenic for phage λ, and modified plasmids carrying the invertible module. In addition, the tightness of the system was evaluated, and the production of a single-chain TCR fragment is presented as an example of successful application of our system.

EXPERIMENTAL AND DISCUSSION

(a) The *att/int* system of Hasan and Szybalski (1987)

Needing a highly repressible and at the same time very flexible and strain-independent expression system we used the pNH series of vectors of Hasan and Szybalski (1987) as the basis of our own constructions. In order to provide the integrase, Hasan and Szybalski (1987) lysogenized their expression strain D1210 (Sadler et al., 1980) with a specially engineered phage λ, to obtain the strain D1210HP. To avoid the lysogenization procedure with this particular phage, which proved in our hands somewhat cumbersome, we constructed a plasmid that could provide the integrase in the same way as the lysogenized phage.

(b) Construction of the integrase plasmid

The plasmid pOU61 (Larsen et al., 1984) was chosen as a vector for the λ integrase. The copy number of this plamid is regulated by a genetically modified R1 *ori* in a temperature-dependent fashion. At a temperature below 30°C the plasmid has a copy number of one, leading to low background expression. At high temperature, the plasmid is amplified, allowing for easy plasmid DNA isolation and high gene expression in the derepressed state. The plasmid is safe-guarded against loss during cell division by a *par* locus (Nordström et al., 1980; 1981).

The integrase gene is expressed under control of the λ p_L promoter and a resident *cI*857-encoded repressor. Integrase expression can thus be induced by a heat shock. To avoid transcriptional interference of the p_L promoter with other parts of the plasmid, an fd terminator was added behind the integrase. To reduce the background expression of the integrase at low temperature further down to the background integrase expression level of the lysogenized phage in D1210HP, an antisense *ompA* promoter (O'Connor and Timmis, 1987) had to be added right behind the integrase gene. This particular promoter was chosen to maintain a steady, medium strength level of antisense transcription (Fig. 1). In the absence of this antisense promoter, enough intergrase is produced even

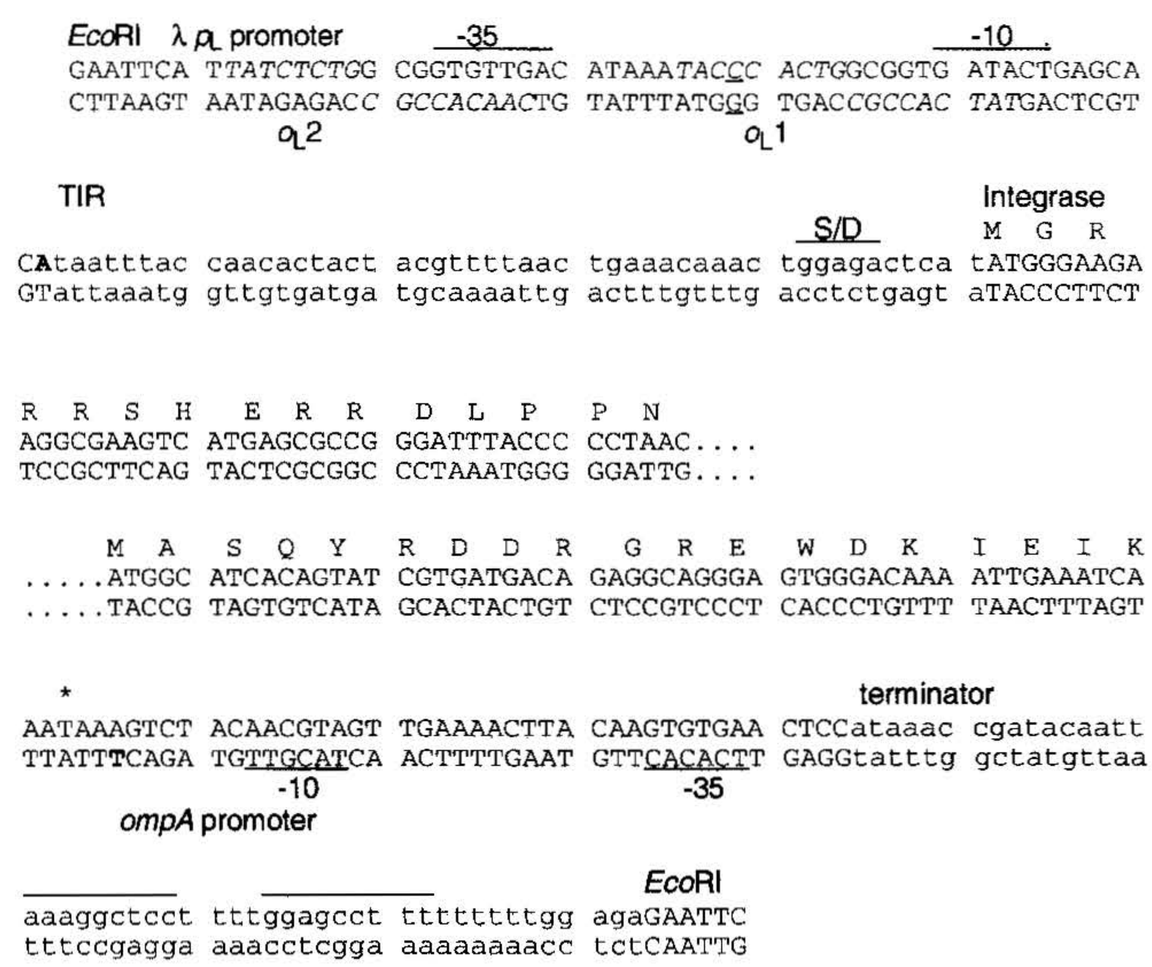

Fig. 1. Integrase expression cassette. The integrase gene is flanked upstream by a λ p_L promoter and by an *atpE* translational initiation region (TIR; in lower-case letters), downstream by an antisense *ompA* promoter and by a bacteriophage fd terminator (in lower-case letters). The −35 and −10 regions of the respective promoters, the Shine-Dalgarno region of the translation initiation region and the hairpin of the terminator are under- or overlined. The asterisk (*) indicates the integrase stop codon. The *tsp* of the two promoters are given in bold letters. The flanking *Eco*RI sites are shown. The p_L promoter of this cassette includes nt 35 647–35 578 of the bacteriophage λ sequence (Sanger et al., 1982), this encompasses the −35 and the −10 region up to the mRNA *tsp* and the o_L1 and o_L2 operators (in italics). The TIR of the *atpE* gene is used as by McCarthy (Schauder et al., 1987). The *ompA* promoter region is the 39 nt in front of the *tsp* (Movva et al., 1981). As terminator, nt 1535–1570 of the bacteriophage fd (Beck and Zink, 1981) are included. **Methods:** The cassette was constructed as follows. The integrase was cloned by PCR from genomic DNA of *E. coli* strain D1210HP (Stratagene, La Jolla, CA, USA) using the oligos 5′-ATGGGAAGAAGGCGAAGT and 5′-TTATTTGATTTCAATTTTGTCC. The p_L promoter, the TIR and the terminator were then added by PCR using oligos 5′-CGGAATTCATTATCTCTGGCGGTGTTGACATAAATACCACTGGCGGTGATACTGAGCACATAATTTACCAACACTACTACGTTTTAACTGAAACAAACTGGAGACTCATATGGGAAGAAGGCGAAGT and 5′-CGGAATTCTCTCCAAAAAAAAAGGCTCCAAAAGGAGCCTTTAATTGTATCGGTTTATTTATTTGATTTCAATTTTGTCC. The PCR product was cloned into the singular *Eco*RI site of pOU61 (Larsen et al., 1984) using the *Eco*RI sites of the long PCR oligos. The antisense *ompA* promoter was added in a second step by PCR using the cassette obtained in the first step as template and the oligos 5′-CGGAATTCATTATCTCTGGCG and 5′-CGGAATTCRTCTCCAAAAAAAAAGGCTCCAAAAGGAGCCTTTAATTGTATCGGTTTATGGAGTTCACACTTGTAAGTTTTCAACTACGTTGTAGACTTTATTTGATTTCAATTTTGTCC. In sequencing the product, an insertion of a C between nt 37 and 38 of the long forward oligo was found. The insertion is underlined in both strands. This mutation seems to be crucial for plasmid stability. A possible explanation might be that increasing the distance between the λ p_L −35 and −10 regions decreases the promoter strength and thus reduces transcriptional interference of the p_L promoter with other parts of the plasmid. The mutation would also be expected to alter the repressor (CI) binding at o_L1. All methods were performed according to standard protocols (Sambrook et al., 1991).

without heat shock that a substantial part of invertible promoter modules is always in the right direction. The complete transcriptional unit was cloned in pOU61 into a unique restriction site between the *bla* gene and the *par* locus, thus obtaining the plasmid pCW107.

(c) Construction of modified pNH plasmids

Since plasmid pCW107 encodes the *bla* gene, a different marker had to be introduced into the pNH plasmid series to allow selection for recombinants containing both plasmids. Since Ap is not an antibiotic favored in fermentation, we decided to alter the high-copy expression plasmid, not pCW107. The genes for kanamycin (*Mlu*I fragment of pBGS18, Spratt et al., 1986) or tetracycline (*Xmn*I, *Sty*I fragment of pTG2, Kadonaga et al., 1984) resistance were inserted into the unique *Bgl*I restriction site within the Ap^R gene. Thus two new plasmids pNH16aKan and pNH16aTet were obtained having the same properties as the pNH series apart from the antibiotic resistance. In the TCR expression plasmids the gene for the *lac* repressor was added at the end of the MCS as well.

(d) Functionality testing on the DNA level

A restriction digest was used to assay inversion. The ability of pCW107 to accomplish the inversion of the promoter module was tested with the non-lysogenic *E. coli* strain JM83 (Yanisch-Perron et al., 1985) transformed with pCW107 in direct comparison with D1210HP (containing the λ lysogen) using pNH16aKan and pNH16aTet. It could be shown that there is no inversion before heat shock in either case (Fig. 2). Whereas inversion after heat shock was complete in D1210HP, in JM83[pCW107] a fraction of the modules was not inverted for unknown reasons (Fig. 2).

In order to investigate the inversion before heat shock (negative control) at higher sensitivity, a PCR based assay was used. Taking one PCR primer outside the invertible module and one within — either hybridizing to the same strand (amplifying only the rearranged plasmid) or to the opposite strand (amplifying only the original plasmid) — two PCR reactions were performed with the DNA of the plasmids pNH16aKan and pNH16aTet, isolated from D1210HP or JM83[pCW107] before heat shock. With both strains tested, a PCR product could be detected which must have been derived from some inverted plasmid modules (data not shown).

It should be noted that the system behaves the same whether Ap was added or not. Thus the *par* locus is sufficient to maintain pCW107 in the cell. For all practical purposes pCW107 is thus functionally identical to integrated λ *xis*⁻ *kil*⁻ *cI* 857, as present in D1210HP: inversion occurs after simple heat shock, the background level of

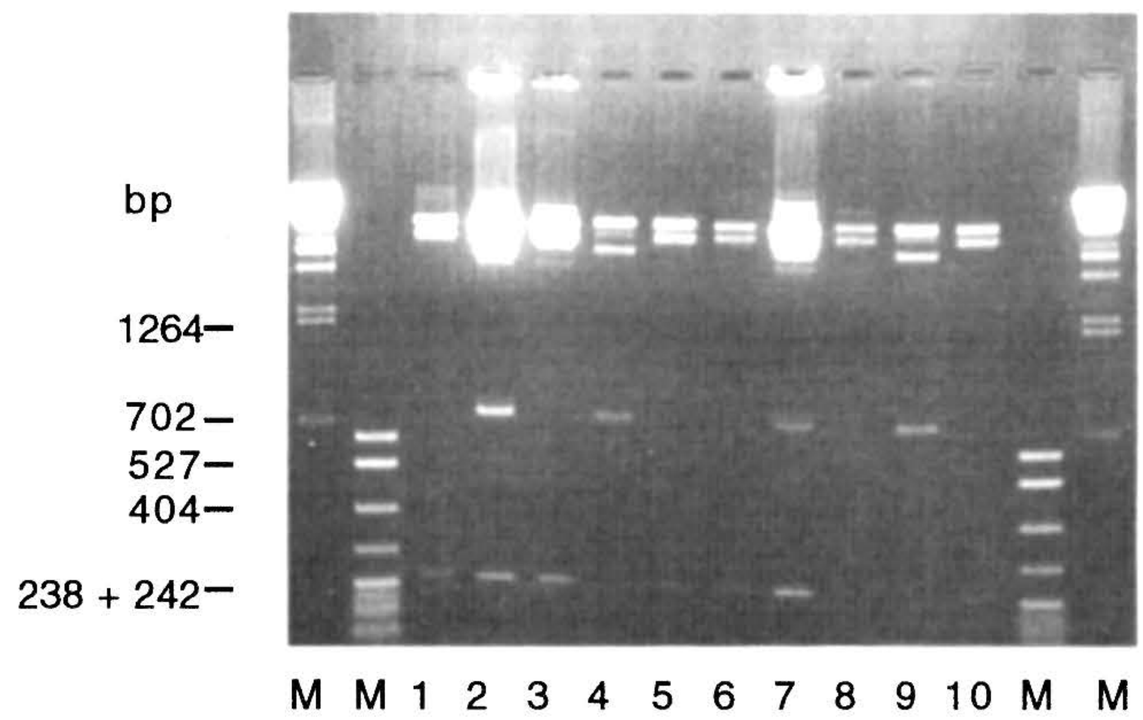

Fig. 2. Inversion of the invertible module by pCW107. Two plasmids were assayed: pNH16aKan and pNH16aTet. D1210HP and JM83[pCW107] were transformed with these plasmids. *E. coli* cells containing pNH16aKan or pNH16aTet were grown at 30°C, half of the culture was then subjected to a heat shock according to the protocol of Hasan amd Szybalski (1987), the other half was kept at 30°C. After plasmid isolation, the DNA was digested with *Nco*I + *Nde*I. The result of 1.5% agarose gel electrophoresis is shown: Lanes 1–5 refer to pNH16aKan, 1 is a control, showing the plasmid DNA that was used for transformation. In lanes 2 and 3 JM83[pCW107] was used as the host for inversion. Three background bands belonging to pCW107 can be seen (at about 500 bp, 550 bp and 850 bp). Lane 2 is with, and lane 3 without heat shock. In lanes 4 and 5, D1210HP was used for inversion. Lane 4 is with, and lane 5 without heat shock. The 238-bp band belongs to the non-inverted module, the band of about 800 bp to the inverted module. The same experiments in the same order for pNH16aTet as plasmid with the module to be inverted are shown in lanes 6–10. Molecular weight markers, denoted M, are the λ *Bst*EII digest and the pBR322 *Msp*I digest (N.E. Biolabs, Beverly, MA, USA). Some relevant marker sizes are indicated.

inversion is very low, and the plasmid can be maintained in the cell without selection.

(e) Functionality testing by producing a toxic TCR fragment

In trying to functionally produce the variable domains of the TCR clone CR15 (Hünig and Bevan, 1982) as a single-chain construct (scTCR) by secretion into the periplasm, the only construct yielding predominantly soluble material was genetically unstable. In contrast, every other construct yielding either partially proteolytically degraded CR15 or predominantly insoluble material was stable. The most likely explanation for the genetic instability of the original construct is that the CR15 fragment is toxic for *E. coli* as soon as it is produced in a soluble, undegraded form (details to be published elsewhere).

In order to obtain a stable construct, producing undegraded, soluble scTCR, a vector was designed which includes all the relevant features of the *lac*-based original expression vector, including the gene for the *lac* repressor, but, as the only difference, the promoter was put within an invertible module. Attempts to produce the scTCR fragment expressed from the invertible promoter vector

in strain D1210HP only led to predominantly insoluble material (data not shown), proving that the choice of the expression strain may be crucial. In contrast, using JM83 [pCW107] as expression strain, scTCR expressed from the invertible promoter plasmid was predominantly soluble (Fig. 3). Thus the same production of soluble, undegraded TCR was observed in JM83 as with the genetically unstable construct, but in a reproducible and stable manner.

(f) Conclusions

(*1*) In the system developed by Szybalski and co-workers (Podhajska et al., 1985; Hasan and Szybalski, 1987) the level of background transcription is controlled by the residual production of the integrase. Moreover, this system has the advantage that the construction and maintenance of the expression vector can be performed in strains which have no integrase-encoding gene (*int*) and thus are completely repressed. The gene to be expressed is preceded by a promoter in the direction opposite to that of the gene to be expressed, reducing the background level of expression to virtually zero during the cloning steps, which proved to be of vital importance in the scTCR experiments (data not shown).

(*2*) Sometimes it may be necessary to finely tune the level of expression, e.g., not to overload the transport apparatus of the host. In addition, the free choice of transcription conditions may be desirable, e.g., when one wishes to express a heterologous protein in the context of its physiological control mechanism. In such a case, it may, e.g., be necessary to include specific repressor- or activator-binding sites. In the invertible promoter system one is completely free in choosing transcription conditions, since the regulation takes place at the level of DNA and not RNA. In addition, one may use any promoter/operator system to be inverted (e.g., the *lac* promoter with the corresponding repressor gene cloned on the same plasmid), thus adding an additional level of control of gene expression. This may serve to uncouple the promoter inversion step from the actual induction of gene expression. Thereby, the influence of a heat shock on protein production can be circumvented.

(*3*) Strain independence may be important in a variety of situations, e.g., robustness may be required in fermentation, protease deficient strains for expression of protease-labile proteins, suppressor strains for transcription of genes containing amber codons and the effect of a gene may be studied in *any* mutant background. The expression system of Hasan and Szybalski (1987) depends on their strain, which was found to be a disadvantage in functionally expressing the *TCR* gene. Our experiments have shown that strain D1210HP (Hasan and Szybalski, 1987) could not substitute for JM83 (Yanisch-Perron

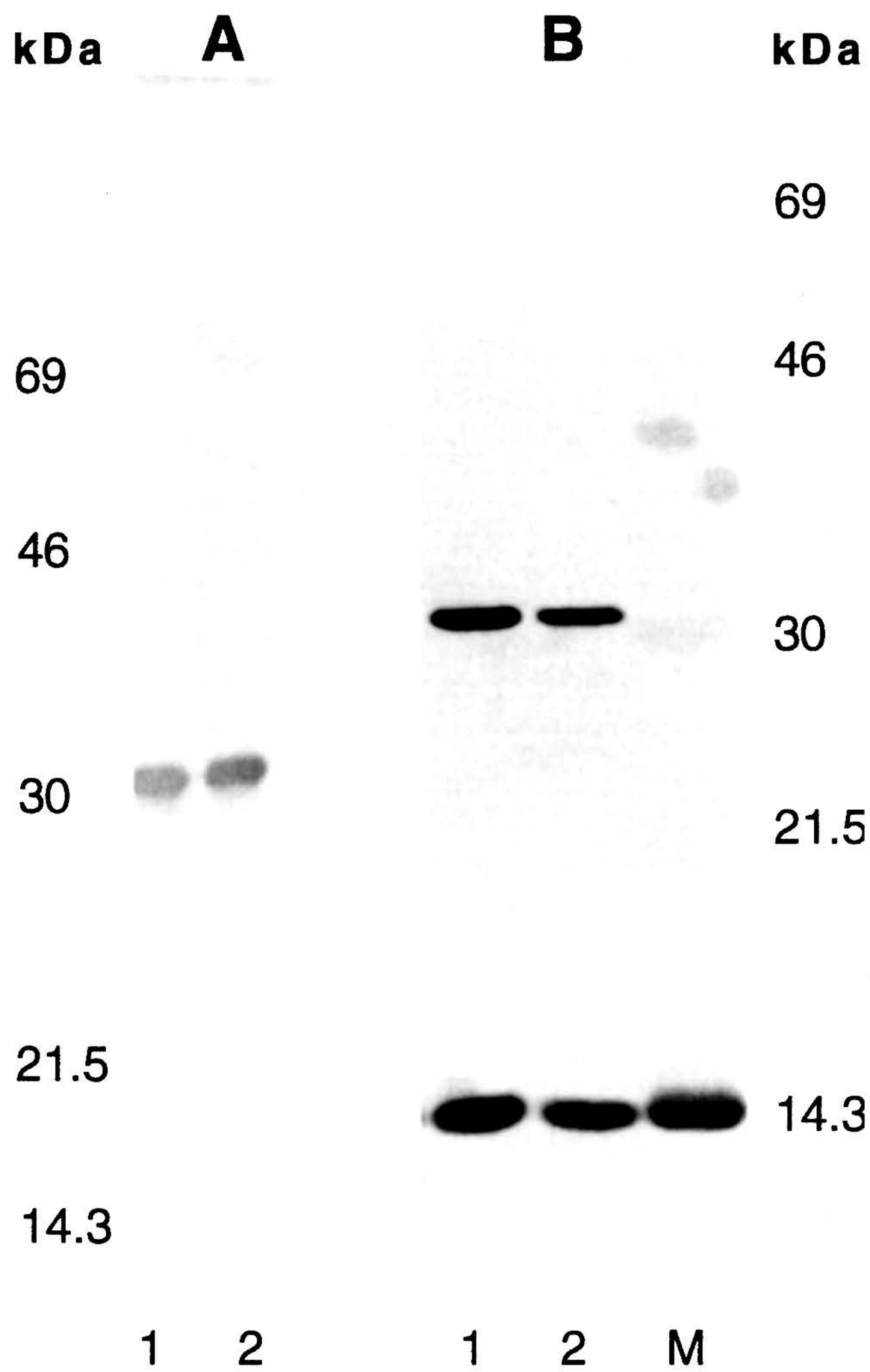

Fig. 3. Western blots of osmotic shock fractions of CR15 scTCR fragments. This figure intends to qualitatively show that the two systems compared behave the same with respect to the production of soluble TCR. The variable domains of the CR15 T-cell receptor (Hünig and Bevan, 1982) were connected with a peptide linker (Lindner et al., 1993) and secreted into the periplasm of *E. coli* using an OmpA signal sequence. Shown are Western blots of the osmotic shock fractions. In **B**, a non invertible system was used. This plasmid rearranged within days on agar plates no longer giving rise to any expression. In **A** the promoter was part of the invertible module in a pNH16a derived plasmid, all other relevant features being equal. In particular, both systems use a *lac* promoter with the *lac* repressor being encoded on the same plasmid. The construct in **A** was stable. Expression strains are JM83[pCW107] in **A** and JM83 in **B**. Lane M contains the lane of the rainbow colored molecular weight marker (Amersham International, England). Lanes 1 and 2 are osmotic shock fractions taken at different time points after induction. **Methods:** As the sequence for an undecapeptide that is part of the c-*myc* oncogene (Munro and Pelham, 1986) was added to the TCR-encoding gene, the scTCR can be detected with a mouse monoclonal antibody directed against the linear c-myc epitope (9E10, Oncogene Science, Uniondale, NY, USA). The secondary antibody in detection is an alkaline phosphatase-conjugated rat anti-mouse antibody (DAKO, Germany). The prominent band of about 30 kDa is the scTCR. Since the secondary antibody cross-reacts with lysozyme used in the cell fractionation, a second band is seen at the bottom of the blot.

et al., 1985). However, plasmid pCW107 confers all functions necessary for the system to a strain of choice in a very simple way. Up to this point, this was the only way to obtain predominantly soluble protein of this particular TCR.

(*4*) In conclusion, we used the invertible promoter principle of Szybalski and co-workers to construct a highly repressible expression system of great flexibility and ease of use. Despite the large number of already published expression systems, our system may be an extension useful for those unlucky, but perhaps not infrequent cases, where the tolerable background level of expression is very low, but a finely tunable expression system is needed.

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