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Award Number: DAMD17-98-1-8270

TITLE: Structural Basis of CDK4 Inhibition by p18INK4

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REPORT DATE: May 2000

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

# REPORT DOCUMENTATION PAGE

*Form Approved*
*OMB No. 074-0188*



| 1. AGENCY USE ONLY (Leave blank) | 2. REPORT DATE | 3. REPORT TYPE AND DATES COVERED |
|---|---|---|
| | May 2000 | Annual Summary (1 May 99 - 30 Apr 00) |

**4. TITLE AND SUBTITLE**
Structural Basis of CDK4 Inhibition by p18INK4

**5. FUNDING NUMBERS**
DAMD17-98-1-8270

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**8. PERFORMING ORGANIZATION REPORT NUMBER**

**9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)**
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

**10. SPONSORING / MONITORING AGENCY REPORT NUMBER**

**11. SUPPLEMENTARY NOTES**

**12a. DISTRIBUTION / AVAILABILITY STATEMENT**
Approved for public release; distribution unlimited

**12b. DISTRIBUTION CODE**

**13. ABSTRACT** ***(Maximum 200 Words)***

The proteins involved in the G1 to S phase transition have been shown to be particularly important in carcinogenesis. Mainly, the disruptions of the pathways involving the pRb and the p53 tumor suppressors have been shown to be common in cancers. Inactivation of the pRb pathway is commonly achieved either by the mutational inactivation of pRb or INK4 tumor suppressors or by the uncontrolled over-expression of Cyclin D1. Cyclin D1 activates CDK4/6 which phosphorylate pRb. The INK4 proteins are specific inhibitors of Cyclin D1-CDK4/6 complexes. We have previously determined the structure of $p18^{INK4c}$. We have prepared more stable single amino-acid mutants of p18INK4c. We are currently conducting urea denaturation experiments to estimate quantitatively the increase in stability. We are also using these mutants in cell cycle assays to determine if the increase in stability correlates with increase in activity.

**14. SUBJECT TERMS**
Breast Cancer, Tumor Supressor, Oncogene, $p18^{INK4c}$

**15. NUMBER OF PAGES**
20

**16. PRICE CODE**

| 17. SECURITY CLASSIFICATION OF REPORT | 18. SECURITY CLASSIFICATION OF THIS PAGE | 19. SECURITY CLASSIFICATION OF ABSTRACT | 20. LIMITATION OF ABSTRACT |
|---|---|---|---|
| Unclassified | Unclassified | Unclassified | Unlimited |

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## Introduction

Disruptions of the pRb or the p53 tumor suppressor pathways appear to be common in cancers. The inactivation of the pRb pathway is commonly achieved either by mutational inactivation of the pRb or INK4 tumor suppressors of the uncontrolled over-expression of Cyclin D1. Cyclin D1 activates Cyclin Dependent Kinase 4/6 (CDK4/6), which phosphorylate pRb[1]. The INK4 family of proteins are specific inhibitors of Cyclin D1-CDK4/6 complexes[2]. A recently identified protein, $p19^{ARF}$, which is an alternatively spliced transcript of the INK4 protein, $p16^{INK4a}$, has been shown to stabilize p53 by preventing its degradation in a DNA damage independent manner[3]. Moreover, $p19^{ARF}$ is activated by pRb, thus linking the two major tumor surveillance pathways[4]. Our proposal focuses primarily on the study of INK4 proteins, we report here progress in specific aims 2 and 3 of our original proposal. We have also initiated side projects to study $p19^{ARF}$ and Cyclin D1. We also report here our attempts to purify these proteins for crystallographic studies. These studies will provide valuable insights into the structural mechanisms underlying the regulation of the pRb and p53 pathways. The insight gained will be invaluable in the design of small molecule drugs that could be used in the treatment of breast cancer.

## Body

### Understanding the mechanism of CDK4/6 inhibition by INK4 proteins through mutants of $p18^{INK4c}$

The focus of our proposal is the study of the INK4 proteins. Here, we report advances in the study of the structural mechanism of inhibition of CDK4/6 by INK4 proteins. As stated in the first annual summary, we solved the structure of $p18^{INK4c}$ (Specific Aim #1) (5, manuscript copies attached to the first annual report) while another research group solved the crystal structures of INK4-CDK6 (6,7). Thus, specific aim #1 and #4 were complete. Several residues that are important in the interaction with CDK4/6 can be inferred from the structures of the free and complexed INK4 proteins. **Using these inferences, we have designed several mutants to test specific aims #2 and #3 (rationale behind these aims was to understand the importance of specific residues in CDK4/6 binding and inhibition).**

The specific aims outlines in the approved statement of work are

1. Determine the structure of $p18^{INK4c}$
2. Prepare mutants of $p18^{INK4c}$ defective in CDK4/6 inhibition
3. Determine the X-ray crystal structure of $p18^{INK4c}$ mutants defective in CDK4/6 inhibition
4. Prepare CDK4-$p18^{INK4c}$ and/or CDK6-$p18^{INK4c}$ complex for structure determination

Using the available crystal structure of free and complexed $p18^{INK4c}$ (5,8), we have designed several mutants to test the importance of a particular residues and overall thermostability of INK4 proteins in CDK4/6 inhibition. The rationale for the mutagenesis and the initial results from the mutagenesis are reported in this annual summary.

Rationale for Mutations

The crystal structure of $p18^{INK4c}$ to 1.95 Å resolution[5] reveals an elongated molecule comprised of five ankyrin repeat units. Each ankyrin repeat contains a beta-strand helix-turn-helix extended strand beta-strand motif that associates with neighboring motifs through beta-sheet, and helical bundle interactions. A large percentage of residues that are conserved among INK4 proteins play important roles in protein stability[5]. Moreover, analysis of the tumor-derived mutations of $p16^{INK4a}$ when mapped on to the structure of $p18^{INK4c}$ seem to show that they play important roles in protein stability[5] (Manuscript attached to first annual report).

Comparison of $p18^{INK4c}$ to the $p18^{INK4c}$ in complex with CDK6 (8) reveals that the structure of $p18^{INK4c}$ is preserved intact without any conformational changes (overall RMS deviation of less than 1.3 Å). This suggests that the thermostability of $p18^{INK4c}$ might be an important factor in its efficacy as an CDK4/6 inihibitor.

The structure of $p18^{INK4c}$ (5) revealed that most hydrophobic residues are buried within the structure forming the bulk of the helical bundle interactions. Similarly, most hydrophilic residues are exposed on the surface forming hydrogen-bonding interactions between repeats. However, we have found several residues that are "misplaced" such as hydrophilic residues that are buried or hydrophobic residues that are exposed. We are presently investigating the role of these residues in the stability and efficacy of the $p18^{INK4c}$ through single point mutations to engineer variants that have increased or decreased thermostability and correlate these mutants to their cell cycle inhibitory function.

Results and Progress

Several structure-based mutations were designed using the structure of $p18^{INK4c}$. These mutations are listed below along with their predicted effect on thermostability. We expect more thermostable proteins to be more efficacious as cell cycle inhibitors. Our goal is to create these mutants, purify them, and characterize them for thermodynamic parameters such as $\Delta G$ and melting temperature relative to the wild type proteins. Finally, we plan to test these mutants *in vivo* using cell cycle assays to assess their efficacy.

| Residue | Rationale for mutation | Mutation | Predicted change in thermostability | Interaction with residues in CDK6[1] | Conservation among INK4 |
|---|---|---|---|---|---|
| Trp 5 | Exposed Hydrophobic | Arg | Increase | No | No |
| Phe 37 | Exposed Hydrophobic | His | Increase | Yes, non-polar side chain contacts | Yes |
| Arg 55 | Buried Hydrophilic | Val | Increase | No | No |
| Phe 71 | Exposed Hydrophobic, Possible inter-repeat contacts | Asn | Increase | Yes, non-polar side chain contacts | No |
| His 75 | Buried polar | Phe | Decrease | No | Yes |
| Phe 82 | Exposed Hydrophobic, Possible inter-repeat contacts | Gln | Increase | Yes, non-polar side chain contacts | Yes |
| Thr 85 | Buried polar | Phe | Decrease | No | Yes |
| Phe 92 | Exposed Hydrophobic, Possible inter-repeat contacts | Asn | Increase | No | No |
| His 108 | Buried polar | Leu | Decrease | No | Yes |

[1] Based on $p18^{INK4c}$ interactions with CDK6 as reported in the ternary complex with viral cyclin [8]

Preliminary experiments have identified several mutants that are more stable and soluble. These include F71N, F82Q, F92N. These residues are hydrophobic and are exposed to the

surface. Moreover, the mutation chosen for these residues are designed to increase inter-ankyrin repeat interactions, thereby increasing thermostability. We are currently conducting Circular Dichroism thermal denaturation experiments to estimate quantitatively the change in stability for all mutants. We will determine the structures of the more stable mutants using X-ray crystallography to explain the change in thermostability. We also expect to perform cell cycle assays with these mutants to determine if the more stable mutants are more efficacious as cell cycle inhibitors. **The results along with any reportable outcomes will be reported in the third (and final) annual report.**

Implications of findings

We are in the process of evaluating the more stable mutants for functional efficacy. If the more thermostable mutants are shown to be more efficacious, then these studies can provide a scaffold for the structure-based design of more thermodynamically stable and more biologically active INK4 proteins. The potential applications of these mutants include applications in gene therapy as well as development of compounds that can stabilize INK4 proteins in the treatment of $p16^{INK4a}$ -mediated cancers, which include breast cancers.

**Other side projects**

In addition to the study of INK4 proteins, we had undertaken the study of human Cyclin D1 and $p19^{ARF}$, both of which are highly relevant to understanding the molecular basis of breast cancer. We hoped that we would be able to purify Cyclin D1 and $p19^{ARF}$ to homogeneity and suitable for crystallographic studies. However, the studies with Cyclin D1 and $p19^{ARF}$ have not been successful to date, some of these studies are reported here.

**Further characterization of human Cyclin D1**

Progress reported in the first annual report

- **Characterization of recombinant human Cyclin D1:** We were successful in solubilizing Cyclin D1 from insoluble fractions of *E.coli* by denaturation in 6M Guanidine HCl and subsequent renaturation by removal of Guanidine HCl by dialysis. However, resulting protein is aggregated as assessed by gel filtration experiments
- **Modelling results suggested that surface cysteines might be the problems:** We identified ten surface cysteines that might be the cause aggregation of protein by the formation of non-specific inter-molecular disulfide bonds. The cysteines targeted were positions 7, 8, 38, 47, 68, 73, 239, 243, 247, and 285. Cysteines were typically mutated to homologous residues in Cyclin D2 or D3 or to serines.

Progress since last annual report

We completed the mutations of ten surface cysteines, however these mutations were not successful in changing the behavior of cyclin D1. The experiments were tried under several conditions such as high salt, and buffer conditions without success. We suspected that the renaturation process might be hindered by the presence of DNA commonly present in inclusion bodies (presence of DNA was confirmed by UV spectroscopy). We purified the denatured ten-cysteine mutant of cyclin D1 prior to renaturation, however, this did not yield monomeric protein. Finally, we tried the addition of iodoacetamide to chemically modify the remaining cysteines and expression of mutants in baculovirus. All these experiments yielded aggregated protein.

Further analysis of our model of cyclin D1 reveals several hydrophobic residues on the surface as well. This suggests that cyclin D1 might be stable only in the context of a complex. To test this hypothesis, we co-expressed cyclin D1 and CDK4 or CDK6 in *E.coli* using a co-expression system of plasmids that we had designed[9]. We found that under several different conditions the co-expression of cyclin D1 and CDK4/6 did not rescue the cyclin D1 proteins (native and the ten-cysteine mutant).

**Further characterization of human $p19^{ARF}$**

Progress reported in the first annual report

- **Characterization of human $p19^{ARF}$:** Human $p19^{ARF}$ has a large number of rare arginine codons. To express human $p19^{ARF}$ we had to construct an artificial gene with optimized codons for expression in bacteria. Moreover, the construct only expressed protein in the context of a GST-fusion protein. However, the fusion protein binds poorly to glutathione and other matrices.

Progress since last annual report

Several approaches were used to attempt the isolation of well-behaved $p19^{ARF}$ or a domain of $p19^{ARF}$. Our attempts to increase the binding of the fusion protein to glutathione and other matrices by changing variables such as salt concentration, buffer pH etc. was unsuccessful. We attempted the denaturation and renaturation of GST-$p19^{ARF}$ obtained from the insoluble fractions. However, this was unsuccessful. We decided to try co-expression of $p19^{ARF}$ with its binding partner mdm2. Upon trying this, we discovered that co-expression does not change the behavior of $p19^{ARF}$. Finally, we tried a smaller construct of $p19^{ARF}$ (the N-terminal 65 residues implicated in mdm2 binding). However, this construct was found to be equally hard to purify as the full length $p19^{ARF}$. Further experimentation with $p19^{ARF}$ have been stopped.

## Key research accomplishments

- Engineered more stable $p18^{INK4c}$ mutants
- Designed (in collaboration with other members of the laboratory) a co-expression system to express protein components of heteromeric complexes in *E. coli*
- Unsuccessfully tried to isolate human Cyclin D1 to homogeneity
- Unsuccessfully tried to purify human $p19^{ARF}$

## Reportable outcomes

Johnston, K., Clements A., **Venkataramani R.N.**, Trievel R.C., Marmorstein R., *Coexpression of proteins using T7-based expression plasmids: expression of heteromeric cell-cycle and transcriptional regulatory complexes.* Protein Expression and Purification 20 (3):435-43, 2000

## Conclusions

Our research primarily revolves around the study of INK4 proteins. INK4 proteins are key regulators of cell cycle progression acting at the G1-S phase transition. INK4 proteins act upstream of pRb tumor suppressor and thereby, control the expression of S-phase specific genes. Two other proteins involved in the p53 and pRb pathways are Cyclin D1 and $p19^{ARF}$. Since, the p53 and pRb pathways has been shown to be important in controlling the proper growth and division of cells, the study of these proteins can yield significant insights into the growth and division of cells. Moreover, the proteins involved in these pathways are commonly mutated or functionally disrupted in cancer. Therefore, the study of these proteins can yield valuable insights into carcinogenesis. We report here studies with the INK4 proteins.

Our original statement of work revolves around the study of INK4 proteins. We have determined the structure of $p18^{INK4c}$ (aim 1) and other investigators have determined the structure of INK4 proteins in complex with CDK (aim 4). These studies suggest that the thermostability of INK4 proteins could directly correlate to their efficacy. Based on this insight, we have designed several mutants to study the impact of the mutation of specific residues on the efficacy of the INK4 proteins (aim 2). We have determined that atleast three mutants – F71N, F82Q, F92N have significantly higher thermostability than the wild-type protein. We are in the process of determining the 3D structures of these proteins (aim 3) as well as testing their efficacy *in vivo*. **We expect to complete aims #2 and #3 and report them in the third and final summary.**

We believe that the study of these mutants designed based on structural information can yield valuable information about the mode of action of the INK4 proteins. Such insights can be directly applied to the treatment of $p16^{INK4a}$ mediated cancers by aiding the design of small molecule drugs or new protein constructs for use in gene therapy.

We have also tried to prepare recombinant Cyclin D1 and $p19^{ARF}$ for crystallographic studies. Our efforts to prepare Cyclin D1 and $p19^{ARF}$ for crystallographic studies have largely been unsuccessful. Despite several different strategies employed to purify these proteins, we have not been able to purify them to homogeneity.

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Protein Expression and Purification **20**, 435–443 (2000)
doi:10.1006/prep.2000.1313, available online at http://www.idealibrary.com on IDEAL®

# Coexpression of Proteins in Bacteria Using T7-Based Expression Plasmids: Expression of Heteromeric Cell-Cycle and Transcriptional Regulatory Complexes

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Received May 29, 2000

**This report describes the development and application of a dual vector coexpression system for the overproduction of heteromeric cell cycle and transcriptional regulatory protein complexes in bacteria. To facilitate these studies we constructed a T7-based expression plasmid, pRM1 that contains an origin of replication derived from p15A, and a gene encoding kanamycin resistance. This expression vector is compatible with ColE1-derived plasmids found in the pET family of T7 expression vectors, which encode ampicillin resistance. It also has the same multiple cloning sites as the pET- derived pRSET vector, allowing easy shuttling between the two expression vectors. Cotransformation of the pRM1 and pET-derived expression vectors into an *Escherichia coli* strain such as BL21(DE3) results in a significant level of coexpression of heteromeric protein complexes. We demonstrate the applicability of combining the pRM1 and pET-derived vectors for the coexpression of cell cycle regulatory components, pRB/E7 and pRB/E1a, and the transcriptional regulatory complexes, SRF/SAP-1 and SRF/Elk-1. We further use the pRB/E1a complex to demonstrate that these coexpressed complexes can be purified to homogeneity for further studies. Use of the pRM1 vector in combination with the pET-derived vectors should be generally applicable for the large-scale coexpression and purification of a wide variety of heteromeric protein complexes for biochemical, biophysical, and structural studies.** © 2000 Academic Press

The preparation of significant amounts of recombinant protein is generally a prerequisite for detailed biochemical, biophysical, and structural studies. There are now many expression systems available for the production of high-levels of recombinant protein in bacterial, yeast, Drosophila, and mammalian systems (1–3). More recently, it has been of interest to study the biochemical and structural properties of multi-protein complexes (4–7). Indeed, many proteins are active only as binary protein complexes. Examples of this include cyclin dependent kinase/cyclin complexes (8) and a host of transcription factor complexes such as the serum response factor (SRF)/ternary complex factors (9, 10). The biochemical and structural analyses of such complexes have largely relied on the ability to reconstitute binary protein complexes from separately prepared recombinant proteins (7, 11–13). Such reconstitution often involves refolding (14, 15) of one or more of the protein components of the complex. More recently, some coexpression systems also have been developed to allow for the direct preparation of binary protein complexes. To date, the most widely used is the baculovirus system (16) in which insect cells are infected with either two independent expression vectors (17, 18) or one vector containing two genes for expression (19). There have been a handful of reports that demonstrate the coexpression of two proteins from the same plasmid in bacteria (20, 21). An expression plasmid has also been reported that, in principle, would allow for the coexpression of proteins in bacteria from two different expression vectors (22).

Here we report the construction of the T7 promoter-based pRM1 vector that is suitable for combination with pET-derived vectors for the coexpression of heteromeric protein complexes in bacteria. There are several factors that make the pRM1/pET-derived vector

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1046-5928/00 $35.00

coexpression system superior to other available systems. First, it makes use of a bacterial expression host, which is still by far the most convenient and widely used system for expressing recombinant proteins. Second, two different expression plasmids allow one to easily mix and match different protein combinations by simply transforming cells with different vector combinations. Third, we have engineered multiple cloning sites for the pRM1 vector to facilitate convenient shuttling of cDNA between pRM1 and the pET-derived vector pRSET.

We demonstrate the general applicability of the pRM1/pET-derived vector system by using it for the coexpression of five different binary protein complexes in bacteria. Two of the complexes are involved in transcriptional regulation and consist of the serum response factor (SRF) with each of two ternary complex factors (TCFs), SRF-associated protein-1 (SAP-1) and Ets-like protein-1 (Elk-1). SRF forms a heteromeric DNA complex with a TCF at the *c-fos* protooncogene promoter to mediate transcriptional activation of that gene (23). Ternary complex formation is mediated by the DNA-binding domain of an SRF dimer (residues 135–222) and by two noncontiguous segments of either SAP-1 or Elk-1. The first segment is a DNA-binding ETS-domain (residues 1–89 in SAP-1 and Elk-1); the second is a B-box region (residues 136–156 in SAP-1 and residues 148–168 in Elk-1) that makes DNA-independent interactions with SRF (24, 25).

Three cell-cycle regulatory complexes are also coexpressed: the retinoblastoma tumor suppressor protein (pRB) with two sizes of adenovirus 5 (Ad5) E1a and with human papillomavirus 16 (HPV 16) E7. pRB is critical in preventing the G1 to S phase transition of the cell cycle and operates, in part, by binding to and repressing E2F transcription factors (26). HPV16 E7 and Ad5 E1a are DNA viral proteins that bind to and abrogate pRB function, causing premature cell cycle progression (27). HPV16 E7 and Ad5 E1a contain homologous regions and have been well characterized (28). However, pRB/HPV16 E7 and pRB/Ad5 E1a complexes have not been extensively characterized biophysically.

Each of these heteromeric complexes produced problematic results when the complexes were reconstituted from separately purified recombinant proteins. For example, SAP-1 and Elk-1 were degraded consistently when expressed and purified individually. In addition, simply mixing purified pRB with purified viral oncoproteins caused aggregation. For these reasons, the coexpression system presented here is essential for the preparation of these protein complexes. Taken together, we believe that the pRM1/pET-derived vector coexpression system reported here provides a convenient and efficient general method for producing heteromeric protein complexes for biochemical, biophysical, and structural studies.

## MATERIALS AND METHODS

### *Materials*

Plasmid pRSET A was purchased from Invitrogen and plasmid pMR103 was a gift from Lynne Reagan (Yale University, New Haven, CT). Plasmids containing cDNA for SRF, SAP-1, and Elk-1 were gifts from Richard Treisman (Imperial Cancer Research Fund Laboratories, London, UK). Plasmids containing cDNA for pRB and E1a were gifts from Robert Ricciardi (University of Pennsylvania Dental School, Philadelphia, PA) as was the plasmid pET-HPV16 E7. Host strains *Escherichia coli* DH5$\alpha$ and BL21(DE3) were purchased from Life Technology Limited. Enzymes were purchased from New England Biolabs, Promega, Boehringer-Mannheim Biochemicals, and Life Technology Limited. IPTG was purchased from American Biorganics, Inc. Oligonucleotides were synthesized at the University of Pennsylvania Cancer Center Nucleic Acid Facility. The QIAfilter Midi and QIEXII Gel Extraction kits, as well as Ni-NTA agarose resin were purchased from Qiagen. Amicon centriprep and centricon protein concentrators with YM-10 MW membranes were purchased from Millipore.

### *Generation of the pRM1 Expression Vector*

Construction of the pRM1 expression vector is outlined in Fig. 1. Unless otherwise noted, recombinant DNA techniques were performed essentially as described (29). A fragment of pRSET A (30,31), spanning positions 2540 to 2941, was amplified by polymerase chain reaction. Primers MCS5, (5′-GCGTGATCACGCCAGATCCGGATATAGTTCC-3′) and MCS3, (5′-ACATGCATGCATGTCGATCCCGCGAAATTAATACGACTC-3′) introduced restriction sites *Bcl*I and *Sph*I, respectively (underlined). The amplified product was digested with *Bcl*I and *Sph*I, heated to 50°C for 5 min and ligated into *Sph*I/*Bam*HI-digested pMR103(22) (*Bam*HI and *Bcl*I produce compatible ends). The insert orientation was verified by restriction digest and the integrity of the manipulated region was confirmed by DNA sequencing at the University of Pennsylvania Department of Genetics DNA Synthesis Facility. Based on the American Type Culture Collection (ATCC) description of pMR103, the following four restriction sites, unique to the multiple cloning region of pRSET were identified as not unique in pRM1: *Hin*dIII, *Nhe*I, *Pvu*II, *Pst*I.

*Generation of DNA Constructs for Recombinant Protein Expression in pRSET A*

cDNA encoding constructs of SRF-1, Sap, ELK-1, and Ad5 E1a were made by PCR using primers which introduced 5′ *Nde*I and 3′ *Bam*HI restriction cleavages sites (underlined.) Primers used for each size construct were: SRF5 (5′-GGATACATATGAAACCGGGTAAGAAGACCCGGGGCC-3′) and SRF3 (5′-CGCGGATCCTTATCTCTGGTCTGTTGTGGGGTCTGAACG-3′) for SRF(135–235); ELK5 (5′-CCGGATACATATGAAAATGGACCCATCTGTGACGCTGTGG-3′) and ELK3 (5′-GGCGGATCCTTATTACTGCGGCTGCAGAGACTGGATGGTG-3′) for Elk(1–170); SAP5 (5′-CCGGATACATATGAAAATGGACAGTGCTATCACCCTGTGGC-3′) and SAP3 (5′-CGCGGATCCTTATTACATGTTCAAAATCTCTGGATAAGAGAC-3′) for SAP(1–158); E1a36_5 (5′-GGGAATTCCATATGAGCCATTTTGAACCACCTACC-3′) and E1a189_3 (5′-GCCGGATCCTTATTCAGACACAGGACTGTAGACAAA-3′) for Ad5 E1a(36–189); E1a114_5 (5′-GGGAATTCCATATGAATCTTGTACCGGAGGTGATC-3′) and E1a189_3 for Ad5 E1a(114–189). cDNA fragments were digested with *Nde*I and *Bam*HI and inserted between the *Nde*I and *Bam*HI sites in pRSET A.

cDNA for pRB (amino acids 376–792) was obtained by PCR using primers RB5 (5′-GCGCTCTAGAACTGTTATGAACACTATCCAACAATTAATG-3′) and RB3 (5′-GCGGGATCCTCAAAACTTGTAAGGGCTTCGAGG-3′), which introduced restriction sites *Xba*I and *Bam*HI, respectively. The PCR product was digested with *Xba*I and *Bam*HI and ligated into *Nhe*I/*Bam*HI-digested pRSET A to create the amino terminal 6× histidine-tagged pRB recombinant (*Xba*I and *Nhe*I produce compatible ends).

*Transfer of SRF and pRB cDNAs from pRSET A to pRM1*

pRSET-SRF was digested with *Nde*I and *Bam*HI and the 303-bp DNA product encoding SRF(135–235) was then ligated into *Nde*I/*Bam*HI-digested pRM1. pRSET-pRB was digested with *Xba*I and *Bam*HI and a 1.3-kb DNA product encoding the ribosomal binding site and the 6× histidine-tagged pRB (376–792) was ligated into *Xba*I/*Bam*HI-digested pRM1.

*Cotransformation*

All proteins were expressed in the *E. coli* strain BL21(DE3). Cells were made competent essentially as described (29) and pRM1-pRB was transformed into competent cells and the cultures were grown on solid or in liquid LB media containing 50 μg/ml kanamycin. These cells were made competent and stored at −70°C. pRSET-Ad5 E1a(36–189), pRSET-Ad5 E1a(114–189), or pET-HPV16 E7 was used to transform the competent cells containing pRM1-pRB. Cells containing both plasmids were grown in LB media containing 50 μg/ml kanamycin (selection for the pRM1 vector) and 200 μg/ml ampicillin (selection for the pRSET vector). The same method was used to cotransform pRM1-SRF with pRSET-SAP or with pRSET-Elk.

*Coexpression*

Colonies were picked from plates containing fresh cotransformed cells and grown in LB media containing 50 μg/ml kanamycin and 200 μg/ml ampicillin at temperatures of 15°C for the pRB complexes and 37°C for the SRF complexes. As the absorbance reached 0.7 $A_{595nm}$, 1 ml of sample was removed, pelleted, and resuspended in denaturant buffer (20 mM Hepes, pH 7.5, 200 mM NaCl, 6 M urea, and 10% glycerol) for inspection by SDS–PAGE. The remainder of the cultures were induced with 1 mM IPTG and incubated for an additional 3–12 h, depending on the growth temperature. One milliliter of these cultures was removed, pelleted, and resuspended in denaturant buffer for inspection by SDS–PAGE. The remaining pellet was harvested by centrifugation and frozen at −20°C. SDS–PAGE was performed as previously described (29). Protein bands were stained with Coomassie brilliant blue. During induction, zinc acetate and magnesium chloride were added to the pRB expressing cultures to a final concentration of 100 μM each.

*Large Scale Expression and Purification of pRB/E1a*

BL21(DE3) cells cotransformed with pRM1-RB(376–792) and pRSET-E1a(36–189) were grown in LB media containing 50 μg/ml kanamycin and 200 μg/ml ampicillin. A single colony was used to inoculate a 400-ml culture that was grown at 30°C to an $A_{595nm}$ of 0.4. 30 ml of this was used to inoculate each of 12 liters warm media containing 50 μg/ml kanamycin and 200 μg/ml ampicillin. These were grown at 37°C until the $A_{595nm}$ reached 0.2, at which time the temperature was decreased to 15°C. When the media was cool and the $A_{595nm}$ was between 0.6 and 0.8 absorbance units. IPTG (1 mM), zinc acetate (100 μM), and magnesium chloride (100 μM) were added. Cultures were grown at 15°C for 16 h. Cells were harvested by centrifugation and stored at −20°C.

Frozen cells were thawed on ice and resuspended in 160 ml sonication buffer (20 mM Hepes, pH 7.5; 200 mM NaCl; 25 mM imidazole, pH 7.5; 100 μM zinc acetate; 10 mM BME.) Cells were lysed by sonication and the insoluble material removed by centrifugation. The supernatant was added to Ni-NTA resin that was equilibrated in sonication buffer and together they were gently stirred at 4°C for 90 min. This slurry was packed in a column and washed with sonication

buffer. Bound protein was eluted from the column over a range of 25–400 mM imidazole. Fractions containing protein were identified using SDS–PAGE stained with Coomassie brilliant blue. These were pooled and DTT was added to final concentration of 20 mM. The sample was concentrated to 1 ml by centrifugation in a Centriprep-10 and purified further by size exclusion chromatography with a Superdex 200 gel filtration column in equilibration buffer (200 mM Hepes, pH 7.5; 200 mM NaCl; 20 mM DTT). Samples were visualized by SDS–PAGE stained with Coomassie brilliant blue. Fractions containing the complex were pooled and concentrated to approximately 30 mg/ml using a Centricon-10. Protein yields ranged from 2.5 to 9 mg of the complex per 12-liter prep.

## RESULTS

### *Construction of the pRM1/pRSET Coexpression System*

A compatible partner plasmid for pRSET and other pET-derived vectors in the coexpression system requires a different *ori* to prevent segregation and ultimate loss of one plasmid in culture (32) and resistance to a different antibiotic to facilitate selection. pMR103, originally designed as a ColE1-compatible expression vector (22), contains a p15A-derived *ori* (33). This factor, combined with its kanamycin resistance gene, makes it a suitable choice for coexpression with pET-derived vectors. However, unique restriction sites for cloning are limited and the medium to low copy number of the p15A *ori* make it more difficult and time consuming to produce and test clones for inserted PCR products.

In order to make the dual vector coexpression system more convenient and efficient, the 400-bp section of pRSET A (31) containing the T7 promoter ($P_{T7}$), the ribosomal binding site (RBS), the multiple cloning site (MCS), and T7 termination sequence (T7 term) was amplified using PCR. This PCR product was inserted directly into pMR103 (22) so that many of the unique restriction sites were retained in pRM1 (Fig. 1). The enzymes *Bam*HI and *Bcl*I were used to digest pMR103 and the PCR product, respectively. This produced compatible cohesive ends for ligation and resulted in a sequence that is no longer recognized by either enzyme. This process removed a superfluous *Bam*HI site from the vector and allowed for the retention of a unique *Bam*HI restriction site in the insert (Fig. 1A). At least eight of the unique restriction enzyme sites from this T7 promoter region and from the multiple cloning site of pRSET are retained in pRM1 (Fig. 1B).

In studying the pRB and SRF protein complexes discussed herein, we first attempted to express and purify the components from pRSET individually. We cloned most of the recombinants using *Nde*I and

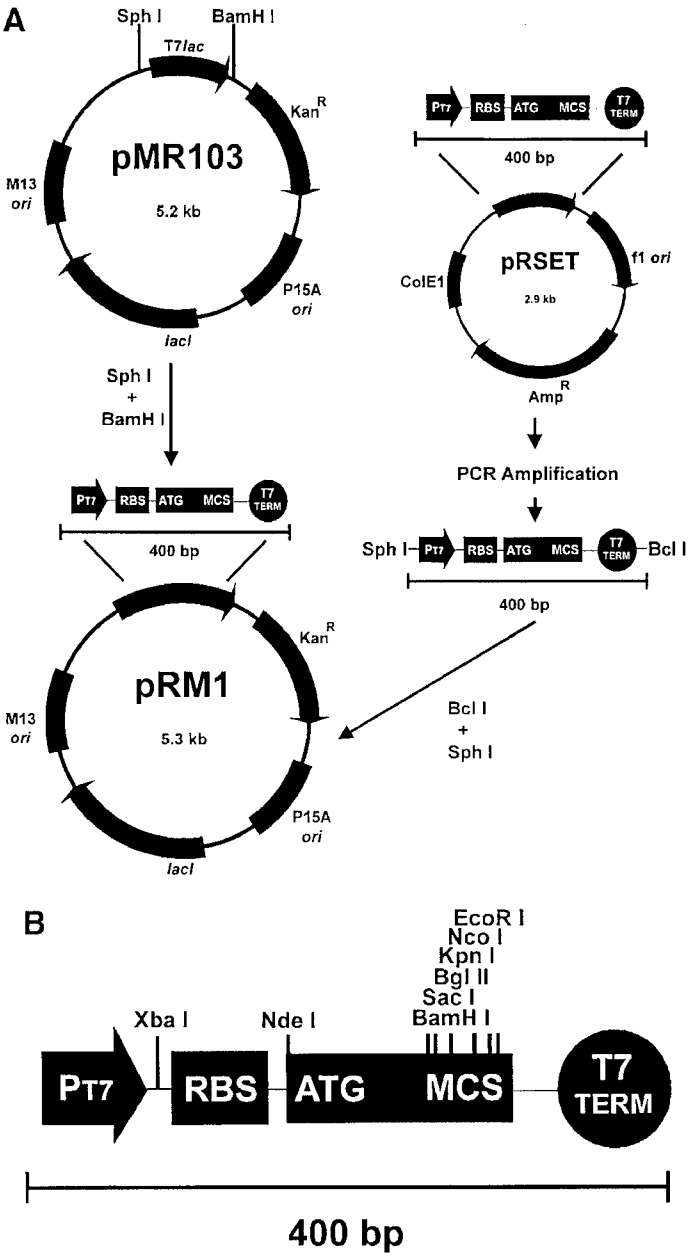


**FIG. 1.** Construction of the pRM1 plasmid. (A) Schematic representation of the construction of the pRM1 plasmid. The 400-bp region of pRSET A (30) containing the T7 polymerase promoter ($P_{T7}$), the ribosomal binding site (RBS), the start codon (ATG) through the multiple cloning site (MCS), and the T7 polymerase termination site (T7 term) was amplified by PCR. Amplification primers introduced *Bcl*I and *Sph*I restriction sites at the 5′ and 3′ ends, respectively. The resulting PCR product was digested with *Bcl*I and *Sph*I, while the pMR103 plasmid (22) was digested with *Bam*HI and *Sph*I to remove the T7 *lac* region. The digested products were gel purified and ligated to form the pRM1 expression vector. (B) Schematic representation of the known unique restriction sites that are shared between pRSET and pRM1.

*Bam*HI restriction enzymes. The one exception was pRB to which we added an N-terminal 6× histidine tag. pRSET is designed to add this to a recombinant if

the cDNA is inserted at the *Nhe*I site down stream of the 6× histidine tag. pRB cDNA was found to be susceptible to digestion by *Nhe*I so a *Xba*I site was designed into the 5′ primer of the pRB construct instead. *Nhe*I and *Xba*I produce compatible cohesive ends making ligation possible so that we could take advantage of the 6× histidine feature of pRSET.

For protein coexpression, we simply transferred either SRF or pRB from pRSET to pRM1. pRSET-SRF had been cloned previously with *Nde*I and *Bam*HI so the SRF cDNA was simply removed from pRSET using these restriction sites and ligated into pRM1. Since *Nde*I can cut the pRB cDNA, this restriction site could not be used to shuttle the 6× histidine-tagged construct from pRSET A to pRM1. However, because both plasmids share many unique restriction sites, an existing *Xba*I site upstream of the *Nde*I site was used and the fragment could be moved directly from one plasmid to the other. The entire DNA sequence of the 6× histidine tag and of the pRB construct was shuttled efficiently from pRSET A to pRM1 using the *Xba*I and *Bam*HI restriction enzymes.

### *Coexpression of Transcriptional Regulatory SRF Complexes*

Previous attempts to overexpress the DNA-binding domain of SRF using the pRSET A overexpression vector resulted in high levels of overexpressed protein that was amenable to purification to homogeneity (34). In contrast, overexpression of either SAP-1 or Elk-1 constructs harboring both the ETS-domain and B-box region consistently yielded a significant proportion of truncated protein product following protein overexpression. The size of the truncated protein product as judged by SDS–PAGE was consistent with proteolytic cleavage within the linker region separating the ETS-domain and B-box regions of the TCF proteins (Yi Mo, K.J., and R.M., unpublished). Therefore, reconstitution of a ternary SRF/SAP-1/DNA complex from separately overexpressed and purified proteins was problematic. We found that a tedious but effective solution to this problem was to mix cells expressing SRF with those expressing a TCF in approximately equal amounts. Surprisingly, although the SRF and TCF proteins did not coelute during cation-exchange and gel-filtration chromatography (presumably due to the relatively weak association of the proteins in the absence of DNA), the purification carried out in this way resulted in no detectable levels of TCF protein degradation (Y. Mo, K.J., and R.M., unpublished). To simplify the preparation of ternary SRF/TCF/DNA complexes, we overexpressed SRF(135–235) in pRM1 for coexpression with the TCF proteins overexpressed in the pRSET A vector. As shown in Fig. 2, coexpression of both SRF(135–235)/SAP-1(1–158) and SRF(135–235)/Elk-1

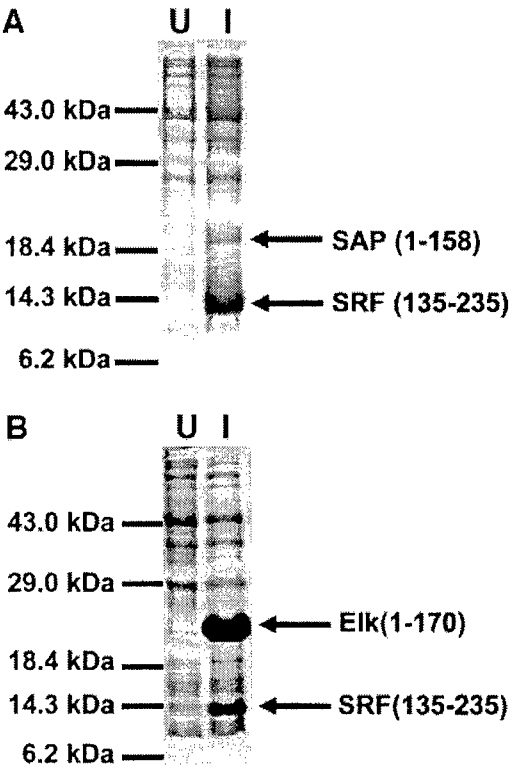


**FIG. 2.** Coexpressed transcriptional regulatory complexes in bacteria. (A) A Coomassie blue-stained 18% SDS–PAGE gel demonstrates that recombinant serum response factor (SRF) and SRF-associated protein-1 (SAP-1) are coexpressed in bacteria. Recombinant SRF (amino acids 135–235) is expressed from the pRM1 vector while recombinant SAP-1 (amino acids 1–158) is expressed from the pRSET A vector. (B) A Coomassie blue-stained 18% SDS–PAGE gel demonstrates that recombinant SRF and Ets-like protein-1 (Elk-1) are coexpressed in bacteria. Recombinant SRF (135–235) is expressed from the pRM1 vector while recombinant Elk-1 (amino acids 1–170) is expressed from the pRSET A vector. All proteins were expressed from the BL21(DE3) bacterial cell line. The lanes labeled with U represent the uninduced samples and the lanes labeled with I represent samples that have been induced with 1 mM IPTG for protein expression.

(1–170) results in high levels of coexpressed proteins. Thus, the pRSET/pRM1 coexpression system produces intact SRF and TCF fragments that should be amenable to ternary complex formation with DNA.

For protein coexpression, simultaneous cotransformation of BL21(DE3) cells with pRM1-SRF and pRSET-SAP-1 vectors was possible but transformation yields were low. Therefore, for each combination of proteins, cells were first transformed with the pRM1 construct, made competent, and then retransformed with the pRSET construct. All protein complexes were coexpressed in bacteria in the presence of ampicillin and kanamycin. Components of transcription factor complexes, SRF(135–235)/SAP-1 (1–158), and SRF(135–235)/Elk(1–170), were grown at 37°C and were coexpressed upon induction with IPTG (Fig. 2).

*Coexpression of pRB Complexes with Viral Oncoproteins*

In order to make significant quantities of pRB/HPV16 E7 and pRB/Ad5 E1a complexes for further characterization, independent bacterial overexpression and purification of pRB(376–792), HPV 16 E7(1–98), Ad5 E1a(114–189), and Ad5 E1a(36–189) were also performed initially. Our attempts at reconstituting such complexes from separately purified recombinant proteins were problematic due to protein instability and persistent precipitation upon mixing relevant protein partners (A.C., K.J., R.M., unpublished). For example, mixing purified HPV 16 E7 with purified pRB caused immediate aggregation in the form of precipitate despite the fact that each of the individually purified components eluted from a gel filtration column as a single nonaggregated species (data not shown). Furthermore, simply mixing HPV16 E7 with pRB was difficult because the molar ratio of HPV16 E7 to pRB in the complex was unknown. In addition, individually purified Ad5 E1a(36–189) was prone to aggregation and eluted from a gel filtration column as a series of oligomers (data not shown), making it unsuitable for pRB complex formation. Therefore, cell cycle regulatory complexes consisting of pRB(376–792)/Ad5 E1a(36–189), of pRB(376–792)/Ad5 E1a(114–189), and of pRB(376–792)/HPV16 E7 were coexpressed (Fig. 3) in bacteria and required low temperature growth conditions for protein solubility. Cultures were started at 37°C but were cooled to 15°C before induction. Expression of these complexes required the addition of IPTG. Since Ad5 E1a and HPV E7 contain zinc binding regions (35, 36), zinc acetate was introduced to the cultures at the protein induction point. In addition, magnesium chloride was introduced to the cultures at the induction point because it was previously demonstrated that pRB requires magnesium for proper refolding from insoluble preparations (37). Cells were not harvested until 12–15 h past time of induction.

For the purification of pRB(376–792)/Ad5 E1a(36–189), we took advantage of the 6× histidine tag on pRB and purified the complex with Ni-NTA resin. We enhanced the resin binding-specificity by loading the protein with the batch method and minimized the nonspecific interactions with the resin by using a buffer containing 25 mM imidazole. Experiments demonstrated that 25 mM imidazole was the maximum concentration that would not interfere with specific protein-resin interactions (data not shown). The complex was further purified using gel-filtration (Fig. 3D). Size-exclusion chromatography also revealed that the two proteins coeluted, indicating that a stable heteromeric pRB/E1a complex was formed. The yield ranged from 0.9 to 1.5 mg of purified pRB(376–792)/Ad5 E1a(36–189) heterodimer per 10-g wet weight cells.

## DISCUSSION

The pRSET/pRM1 coexpression vectors fit four criteria that are necessary for a convenient two vector bacterial coexpression system. First, both vectors are compatible with a T7-promoter/T7-polymerase protein expression system. Second, each vector has a different antibiotic resistance gene for selection. Third, the two vectors have compatible origins of replication to prevent segregation and ultimate loss of one plasmid in culture (32). Finally, both vectors have useful multiple cloning sites that are compatible with each other and facilitate the shuttling of DNA constructs between the two vectors.

The variety of unique sites shared by the two plasmids allows for simple cDNA shuttling between pRSET A and pRM1, a process which is less time consuming and more efficient than generating new inserts by PCR amplification. This was especially useful for our studies because the protein fragments we were interested in coexpressing from pRM1 had previously been subcloned into pRSET A. The shared unique restriction sites between the two vectors (Fig. 1B) simplified cDNA shuttling and allowed for a direct "cut and paste" of the sequence of interest from pRSET A into pRM1.

In order to use this coexpression system, protein complexes should have three main characteristics. First, both recombinant proteins should express well in bacteria. The overall expression levels of recombinant proteins during coexpression were typically in the range of one half to one fourth that of the single recombinant protein expression levels from either plasmid. In addition, we found that the pET-derived vector generally expressed more protein than did pRM1 during co-expression (Fig. 2B), which could be related to the copy numbers of the two plasmids, high for ColE1 in pRSET and medium-low for p15A in pRM1. This is consistent with levels of protein expression in bacteria being proportional to the availability of protein-encoded genes in the cell (38). A second requirement for the complex is that the recombinant proteins should have lower than micromolar dissociation constants so that proteins can be copurified by standard chromatographic techniques. Finally, a copurification scheme should be developed which is easily accomplished if one of the components of the complex contains a feature designed for affinity chromatography.

We first used this system in preparation for structural studies of transcriptional regulatory complexes involving SRF, SAP-1, and Elk-1. Attempts to purify the components individually were unsuccessful. SRF was easily purified but both SAP-1 and Elk-1 degraded during purification. We had some success expressing SRF and TCFs in separate cultures, mixing the lysates, and copurifying the protein complexes, but this process

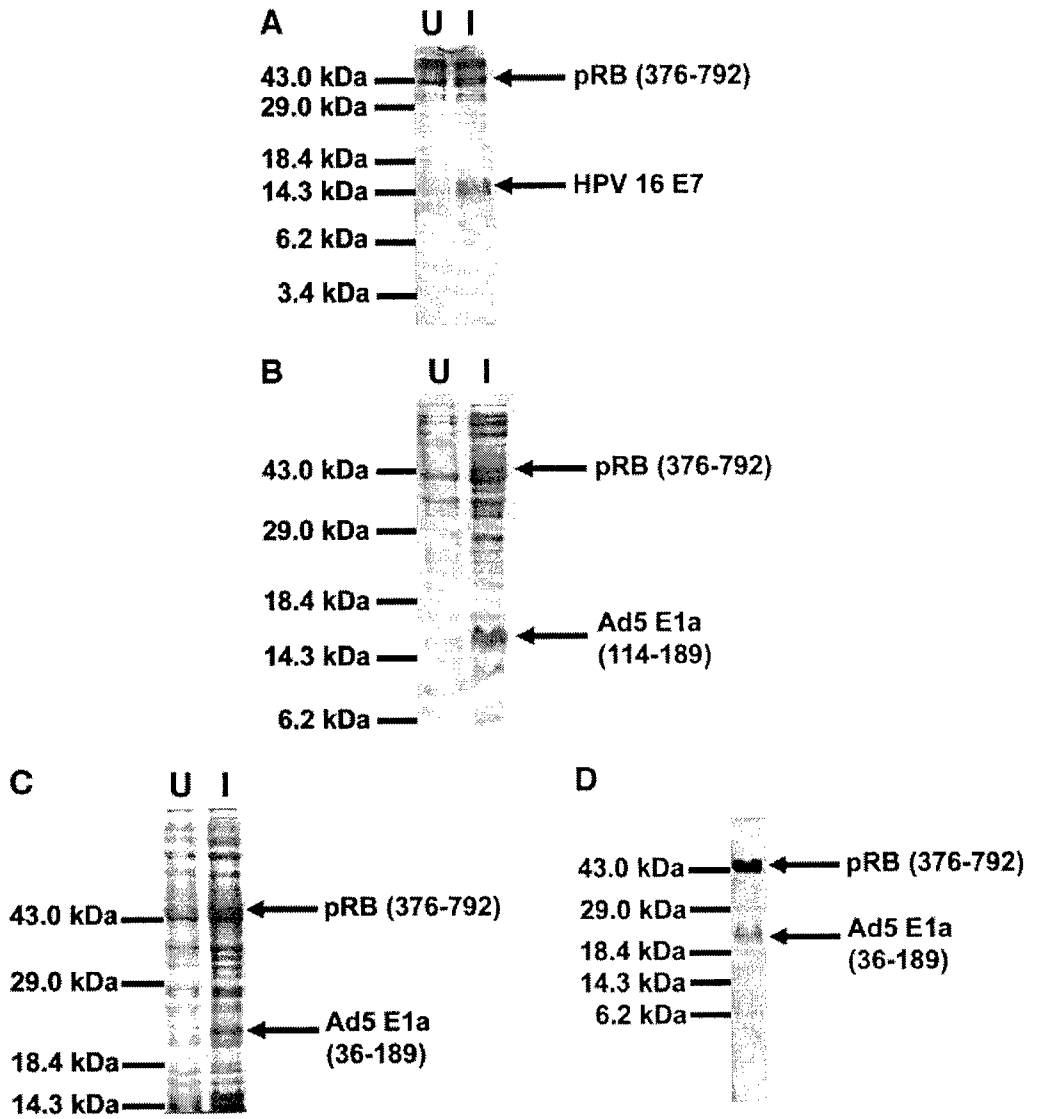


**FIG. 3.** Coexpressed cell-cycle regulatory complexes in bacteria. (A) A Coomassie blue stained 22% SDS–PAGE gel demonstrates that the recombinant retinoblastoma tumor suppressor protein (pRB) and human papillomavirus 16 E7 (HPV16 E7) are coexpressed in bacteria. An amino terminal 6× histidine-tagged construct of pRB (amino acids 376–792) is expressed from the pRM1 vector and HPV16 E7 is expressed from the pRSET A vector. (B) A Coomassie blue-stained 18% SDS–PAGE gel demonstrates that recombinant pRB and Ad5 E1a(114–189) are coexpressed in bacteria. pRB(376–792) is expressed from the pRM1 vector while Ad5 E1a(114–189) is expressed from pRSET A. (C) A Coomassie blue-stained 18% SDS–PAGE gel demonstrates that recombinant pRB and Adenovirus 5 E1a (Ad5 E1a, amino acids 36–189) are coexpressed in bacteria. pRB(376–792) is expressed from the pRM1 vector, while Ad5 E1a(36–189) is expressed from pRSET A. All proteins were expressed from the BL21(DE3) bacterial cell line. The lanes labeled with U represent the uninduced samples and the lanes labeled with I represent samples that have been induced with 1 mM IPTG for protein expression. (D) A Coomassie blue-stained 18% SDS–PAGE gel demonstrates a protein peak fraction of the pRB(376–792)/Ad5 E1a(36–189) complex from a Superdex 200 gel filtration column.

was inefficient. In order to simplify the preparation of ternary SRF/TCF/DNA complexes, we moved the gene coding SRF(135–235) from pRSET into pRM1 and co-expressed it with each of the TCF proteins. Coexpression of SRF/SAP and of SRF/Elk successfully produced both components of each complex (Fig. 2). Thus, the pRSET/pRM1 coexpression system produces intact SRF and TCF fragments that should be amenable to ternary complex formation with DNA. Moreover, these studies suggest that the coexpression and subsequent purification of other heteromeric transcriptional regulatory proteins should also be feasible.

Using this system, we were also able to coexpress pRM1-pRB (376–792) with pET-HPV16 E7 (Fig. 3A) or with pRSET-Ad5 E1a (Figs. 3B and 3C) in bacteria. Since mixing individually purified components caused precipitation, this coexpression system was utilized to promote the optimal protein conformation for complex formation immediately after expression. This system enabled the formation a pRB/viral protein complex

with proper stoichiometry. In addition, the preparation time for the pRB/viral protein complexes was minimized with this coexpression system because both proteins were purified simultaneously.

In summary, we have developed a bacterial expression vector that may be combined with pET-derived vectors for the coexpression of heteromeric protein complexes in bacteria. The new vector, pRM1, contains an *ori* compatible with that in the pET-derived vectors, a kanamycin resistance gene for selection and, most importantly, a variety of unique restriction sites that are shared with pRSET for efficient shuttling of constructs between pRSET A and pRM1. This two vector coexpression system is distinct from other coexpression systems because it provides the capacity to efficiently mix and match coexpressed protein partners in bacteria. This allows for the effective production of a variety of protein complexes for detailed biochemical, biophysical, and structural studies.

## ACKNOWLEDGMENTS

The authors thank Yi Mo for useful discussions. This work was supported by an ACS (RPG-98-235-GMC) grant and a NIH (GM-52880) grant to R.M., a U.S. Army Breast Cancer Research predoctoral fellowship to A.C. (DAMD17-97-1-7063), a U.S. Army Breast Cancer Research predoctoral fellowship to R.N.V. (DAMD17-98-1-8270), and a Howard Hughes predoctoral fellowship to R.C.T. (70109-522202).

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