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Point Mutational Inactivation of the Retinoblastoma Antioncogene

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The retinoblastoma (Rb) antioncogene encodes a nuclear phosphoprotein, p105-Rb, that forms protein complexes with the adenovirus E1A and SV40 large T oncoproteins. A novel, aberrant Rb protein detected in J82 bladder carcinoma cells was not able to form a complex with E1A and was less stable than p105-Rb. By means of a rapid method for the detection of mutations in Rb mRNA, this defective Rb protein was observed to result from a single point mutation within a splice acceptor sequence in J82 genomic DNA. This mutation eliminates a single exon and 35 amino acids from its encoded protein product.

GENETIC ANALYSIS OF RETINOBLASTOMAS and osteosarcomas has revealed a cellular gene, *RB-1*, whose homozygous inactivation is a critical determinant of tumorigenesis (*1*). Because the absence of functional Rb alleles correlates with tumor formation, it is widely assumed that this gene acts to constrain the growth of normal cells. Its loss is thought to remove a barrier to cell proliferation, leading in turn to the growth of tumor cell clones.

Recently we and others reported isolation of a molecular clone of a gene that has many of the expected properties of the Rb gene (*2–4*). For example, structural analysis of chromosomal deletions found in a small number of retinoblastomas and osteosarcomas led to the conclusion that this cloned gene, and not neighboring genes, is the common target of the random chromosomal deletions that triggered these tumors. By means of Southern blot analysis, deletions of the Rb gene have been found in only 15 to 40% of retinoblastoma tumors examined to date (*2–5*). It has been assumed that the Rb gene may be altered in some or all of the remaining tumors in subtle ways that are not detectable by Southern blotting but, nevertheless, suffice to inactivate the function of the Rb gene. We report here just such a mutation, discovered through analysis of the Rb-encoded proteins found in a number of human tumor cell lines.

The Rb gene encodes a nuclear phosphoprotein of approximately 105 kD, p105-Rb, that is associated with DNA-binding activity (*6, 7*). Recent experiments have suggested that this protein may also be involved in alternative tumorigenic mechanisms that do not depend upon mutational alteration in the Rb gene itself. Specifically, we have found that in adenovirus-transformed cells, the virus-encoded E1A oncogene proteins are complexed with as many as ten host cell proteins. Among these is a protein of 105 kD that we have identified as p105-Rb (*7*). Detailed studies of E1A mutants have established that mutations disrupting the binding of E1A to p105-Rb ablate the transforming powers of the E1A oncoprotein (*8, 9*). We concluded that the same protein (p105-Rb) found to be absent from spontaneously arising human retinoblastomas is also perturbed by E1A proteins as part of adenovirus-mediated transformation. Evidence from one of our laboratories as well as others has established that p105-Rb is a common target for protein binding by two other oncoproteins, SV40 large T antigen and the E7 protein of human papillomavirus (*10, 11*).

We have used the formation of the E1A:p105-Rb protein complex as an assay for the presence and function of p105-Rb. To this end, normal and tumor cell lines were infected with human adenovirus type 5. Any resulting E1A:p105-Rb complexes were detected in these cells via immunoprecipitation with a monoclonal antibody, M73 (*12, 13*), that reacts specifically with the E1A oncoproteins. Such a test had shown the presence of E1A:p105-Rb complexes in most infected tumor cell types and its absence from several adenovirus-infected retinoblastoma cell lines (*7*). This absence was in consonance with the loss of functional p105-Rb from retinoblastomas. Unexpectedly, we also were unable to find E1A:p105-Rb complexes in adenovirus-infected human J82 bladder carcinoma cells (Fig. 1A). Changes in the Rb gene with loss of p105-Rb have not previously been associated with bladder carcinomas.

Use of a monoclonal antibody, C36, specific for the Rb protein (*7*) and two independent rabbit anti-peptide sera reactive with the Rb protein (*7*) made it possible to determine whether this protein was present in the cells of the J82 tumor line. Immunoprecipitations with C36 monoclonal antibody (Fig. 1A), antiserum prepared against an internal synthetic peptide, #147, or antiserum #144 (which reacts with the COOH-terminus) all precipitated an Rb-related protein of approximately 102 kD (Fig. 1B). The C36 monoclonal antibody also precip-

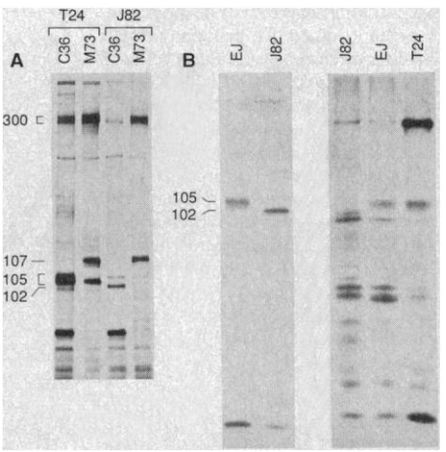

Fig. 1. Immunoprecipitation of Rb protein and E1A:Rb protein complexes from bladder carcinoma cells. (**A**) Cultures of J82 and T24 bladder carcinoma cells were infected with adenovirus type 5 and 10 hours later incubated for 3 hours with [^{35}S]methionine. Cell lysates were prepared as described (*7*) and immunoprecipitated with monoclonal antibodies against Rb protein (C36)(*7*) or adenovirus E1A proteins (M73) (*12*). Immunoprecipitates were resolved by electrophoresis for 2.5 hours on an 8%–4% discontinuous SDS-polyacrylamide gel, processed for fluorography, and exposed for 2 days at −70°C. Indicated are the sizes of the Rb proteins precipitated from J82 and T24 cells, as well as two cellular proteins, 300 kD and 107 kD, that are coprecipitated by M73. (**B**) Cultures of J82, EJ, and T24 bladder carcinoma cells were incubated with [^{35}S]methionine and cell lysates prepared as in (A). Immunoprecipitations of Rb protein were performed with two independently derived rabbit sera to the synthetic peptides characterized previously (*7*). Rabbit sera #147 (reactive with an epitope internal to the Rb protein) was utilized in the two leftmost lanes, and rabbit sera #144 (reactive with the Rb COOH-terminus) in the rightmost three lanes.

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itated a phosphorylated form of the 102-kD Rb protein that migrates at a rate corresponding to 104 kD. Treatment of such C36 immunoprecipitates with potato acid phosphatase reduces this 104-kD phosphorylated species to its nonphosphorylated 102-kD form (*13*). Hence, although present in adenovirus-infected J82 cells, the aberrantly sized 102-kD Rb protein and its phosphorylated counterpart were unable to complex with E1A.

The Rb-related protein of J82 cells migrated during electrophoresis with the mobility of a protein having a mass several kilodaltons smaller than that of the wild-type p105-Rb (Fig. 1B). We suspected that this apparently altered size reflected a lesion that also prevents this protein from associating with the E1A oncoproteins. The more rapid migration of the J82 Rb protein could result from several causes, one being deletion of a portion of the coding sequence of the Rb gene. Any such deletion, however, would have to leave downstream coding sequences intact and in-frame with upstream sequences since antiserum #144, which recognizes an epitope encoded by the Rb COOH-terminus, was able to precipitate the J82 Rb protein.

Further analysis showed the defectiveness of this novel 102-kD Rb protein to be manifest in more than its inability to bind E1A proteins. The half-life of this mutant form of p105-Rb was less than 3 hours as measured in a pulse-chase experiment (Fig. 2). This is in contrast to a half-life of more than 6 hours that we have observed with the wild-type p105-Rb in bladder carcinoma cells.

We also determined whether the novel 102-kD Rb protein had lost the associated DNA-binding activity attributed to wild-type p105-Rb (*6*). [^{35}S]Methionine-labeled lysates from J82 cells were bound to double-stranded DNA cellulose and proteins eluted with increasing concentrations of salt. Resulting fractions were assayed for Rb protein by immunoprecipitation. Both the wild-type p105-Rb and the J82 Rb protein exhibited identical elution profiles (Fig. 3) suggesting that loss of E1A-binding activity is not associated with a concomitant loss of DNA-binding activity.

To assess the nature of the genetic defect that underlay the alterations of the Rb protein in J82 cells, we analyzed the RNA of J82 carcinoma cells by means of a recently developed hybrid protocol of the polymerase chain reaction (PCR) and cDNA synthesis techniques (*14*). A first strand of cDNA was made by reverse transcription with oligo(dT) as primer. Appropriate oligonucleotide primers were prepared that delineated three regions of amplification that together encompassed the entire p105-Rb reading frame (Fig. 4B). Synthesis of the second strand of the cDNA and subsequent amplification was driven by PCR reactions with these synthetic primers. Reaction products were resolved by gel electrophoresis and visualized by ethidium bromide staining. One PCR amplification utilizing primers R55 and R33 yielded a truncated product, indicating a deletion associated with the second third of the reading frame between nucleotides 1873 to 3046. A 1200-bp fragment seen upon analysis of normal human RNA and the RNAs of several retinoblastomas was truncated when J82 RNA was used as template for PCR amplification (Fig. 4A). This deletion was more precisely defined by additional PCR reactions in which primers homologous to sequences within this novel fragment were used and by cloning and sequencing of the PCR reaction products. Thus, PCR reactions with primers R55 and R37 produced an aberrantly sized DNA fragment when RNA from J82 cells was used as a template, whereas primers R57 and R33 produced PCR fragments of wild-type size (Fig. 4A). These latter amplification reactions delimited a deletion in J82 RNA of approximately 100 nucleotides between bases 1872 to 2410.

Dideoxynucleotide sequence analysis of the cloned PCR fragment amplified by primers R55 and R37 showed the absence of a 105-nucleotide stretch from J82 RNA that is normally observed in the Rb cDNA sequence (Fig. 4B). This deletion was confirmed by dideoxynucleotide sequencing of 24 independent M13 phage clones derived from three independent PCR reactions with primers R55 and R37. All resulting phage showed an identical 105-nucleotide deletion in J82 RNA. This "in-frame" deletion results in the elimination of 35 amino acid residues, from positions 697 to 731, but allows continued translation of distal sequences. All other regions of J82 RNA appeared normal in size in parallel PCR reactions with other synthetic primers. The

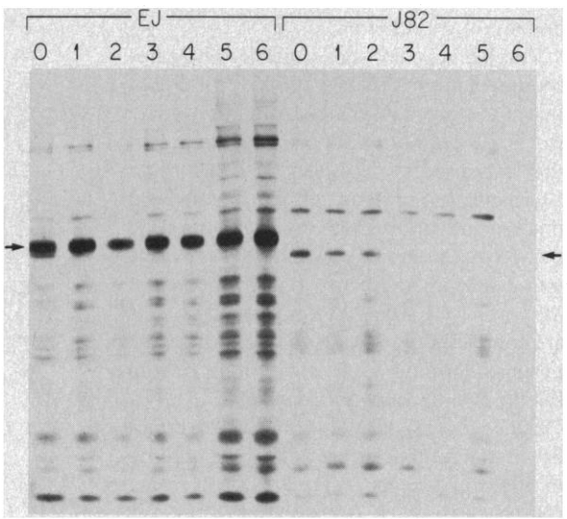

Fig. 2. Pulse-chase analysis of wild-type and mutant Rb proteins from bladder carcinoma cells. Cultures of EJ and J82 cells were incubated with [^{35}S]methionine for 1 hour and then washed extensively with medium containing cold methionine. Cell lysates were prepared as in Fig. 1 after no further incubation (0 hours) and each hour thereafter for 6 hours. Immunoprecipitations using rabbit sera #147 were performed and processed as in Fig. 1. Arrows indicate position of wild-type (EJ) and mutant (J82) Rb proteins.

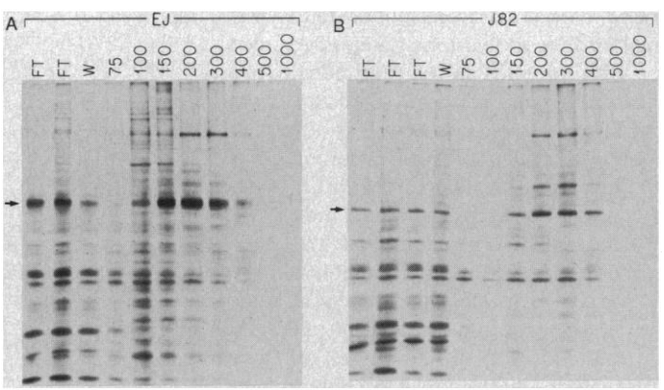

Fig. 3. Associated DNA-binding activity of wild-type and mutant Rb proteins from bladder carcinoma cells. Cultures of EJ (**A**) and J82 (**B**) cells were incubated with [^{35}S]methionine and cell lysates prepared as in Fig. 1. EJ and J82 lysates were diluted with lysis buffer lacking NaCl to a final concentration of 50 m*M* NaCl (2-ml final volume for EJ lysate, 3-ml final volume for J82). Lysates were passed through 1-ml columns of double-stranded DNA-cellulose (Pharmacia) previously equilibrated with lysis buffer containing 50 m*M* NaCl. Flow-through (FT), 50 m*M* NaCl wash (W) fractions, and then successive 1-ml fractions of eluted proteins were collected with stepwise increases in NaCl concentration from 75 to 1000 m*M*. Collected fractions were immunoprecipitated with rabbit sera #147 and precipitates processed as in Fig. 1. Arrows indicate positions of wild-type (EJ) and mutant (J82) Rb proteins.

35–amino acid deletion thus appears to underlie the increased electrophoretic mobility of the J82 Rb protein. We presume that this deletion also prevents complex formation with the E1A oncoproteins. We cannot, however, exclude the possibility that point mutations that would have been overlooked by our analysis do not also prevent J82 Rb:E1A complex formation.

We next sought to determine the chromosomal lesion that causes this deletion of sequences normally present in Rb mRNA. We have recently defined the detailed structure of the Rb gene; it spans 200 kb of DNA and encompasses 27 separate exons (*15*). These exons, together with flanking intronic sequences, have been subjected to detailed sequence analysis (*16*). Comparison of the observed 105-bp cDNA deletion with the detailed structure of the Rb gene revealed a precise congruence between the deleted sequences and those present in exon 21. We concluded that the deletion in the reading frame was attributable solely to the absence of this exon in the processed Rb mRNA present in J82 cells.

When J82 genomic DNA was surveyed with appropriate cDNA and genomic probes in Southern blot analysis, a normal pattern of restriction fragments was observed (*17*). This indicated that the absence of the exon in the mRNA was not due to deletion of corresponding chromosomal sequences and prompted us to examine this region of J82 chromosomal DNA in greater detail. Using a panel of oligonucleotide primers and PCR-mediated amplification described elsewhere (*18*), we examined exon 21 of the J82 Rb gene together with flanking intron sequences; J82 genomic DNA was the template. Direct sequencing of the reaction products revealed a point mutation in the splice acceptor site at the 5′ end of exon 21. No PCR fragments encoding wild-type splice acceptor sequences were recovered in these amplification reactions, indicating that J82 cells are either homozygous or hemizygous for the mutant allele.

The observed mutation converts a canonical splice acceptor sequence, 5′-TTTATTTACTAG-3′, present in the wild-type allele, into the sequence 5′-TTTATTTACTGG-3′. No similar mutant allele was recovered when genomic DNAs from 18 unrelated individuals were sequenced in this region (*18*). Presumably, the point mutation found in the Rb gene of J82 cells abrogates normal splicing at this site. As a consequence, exon 21 is discarded during the biogenesis of the Rb mRNA in these cells, resulting in the fusion of exon 20 directly to exon 22. This leads in turn to the production of a truncated Rb protein. In contrast to wild-type p105-Rb, this aberrant, destabilized protein is unable to bind E1A oncoproteins. In addition, further analysis of the mutant J82 Rb protein has shown that it also fails to complex with SV40 large T antigen (*17*). It is likely that this defective Rb protein is also deficient in carrying out its normal functions within the cell.

These findings extend our understanding of the involvement of the Rb gene in tumorigenesis. It shows, as long suspected, that subtle lesions in the 200-kb Rb genomic sequence can serve as loss-of-function mutations. Similar lesions have been documented in globin alleles associated with thalassemia, type I procollagen genes, and in the dihydrofolate reductase gene (*19*). Only a minority of retinoblastomas have Rb alleles that show gross alterations in gene structure, suggesting the presence of more subtle lesions in the remaining tumors. The point mutation reported here in a bladder carcinoma cell line is an example of such a lesion. Recently, examples of nucleotide substitutions in Rb mRNA have been reported in Rb tumor cells (*20*). The present work extends observations that mutant Rb alleles may be found in tumors other than retinoblastoma and osteosarcoma (*21*). In this vein, recent reports document deleted and rearranged Rb genes in small cell lung cancers and mammary carcinomas (*22*).

It is likely that the observed point mutation and consequent p105-Rb defect conferred selective growth advantage on the cells that spawned the J82 tumor or derived cell lines. However, as normal tissue is not available from the patient from which the J82 bladder carcinoma line was derived, we

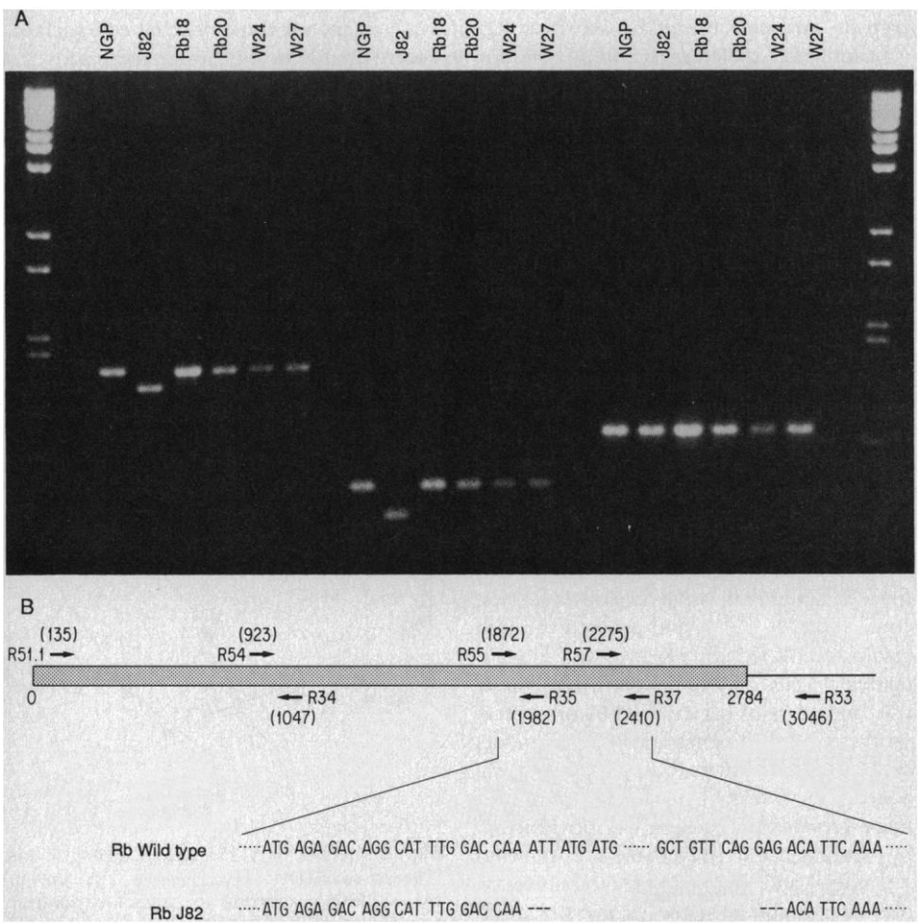

Fig. 4. Wild-type and mutant DNA fragments amplified and sequenced from total cell RNA with Rb synthetic primers and the polymerase chain reaction technique. (**A**) One microgram of total cell RNA was prepared from J82 cells, NGP human neuroblastoma cells, and four retinoblastoma cell lines (*24*) and PCR amplified fragments were synthesized essentially as described (*14*) with oligo(dT) and synthetic oligonucleotides. After 40 cycles of amplification, DNAs were separated by electrophoresis through a 1% agarose gel containing ethidium bromide and visualized by ultraviolet illumination. The left set of amplified fragments utilized primers R55 and R33, the center set utilized primers R55 and R37, and the right set utilized primers R57 and R33 [see (B) for map location of primers]. Bst EII-cut λ DNA appears in outside lanes as molecular weight standards. (**B**) Schematic diagram of Rb cDNA, location of oligonucleotide primers utilized for PCR amplification, and deduced sequence of J82 and wild-type cDNA in region of deletion. Sense of oligonucleotide primers is indicated by arrows and their positions along the cDNA are given in nucleotides numbered from the first nucleotide of coding sequence. The coding region of the Rb cDNA is designated by a hatched box.

can only speculate as to the causative influence of this defective Rb protein. The J82 point mutation we have documented leads to the synthesis of a protein that fails to bind the E1A and SV40 large T oncoproteins yet still retains an associated DNA-binding activity. If this latter activity is important in the normal physiology of p105-Rb, then we must assume that DNA binding alone may not be sufficient for the antineoplastic function of Rb.

Our analysis of the J82 Rb gene expands the catalog of genes in which point mutations create potentially tumorigenic proteins. To date, these have only been well documented in the *ras* and *neu* oncogenes (*23*). Use of the adenovirus E1A complex assay, PCR technology, and a detailed map of the Rb gene made this analysis possible in a relatively short period of time and indicates the possibility of rapid molecular analysis of mutant Rb alleles encountered in the future (*16, 18*).

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Epithelial Cell Surfaces Induce *Salmonella* Proteins Required for Bacterial Adherence and Invasion

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Salmonella **bacteria are capable of entering (invading) and multiplying within eukaryotic cells. Stable adherence to and invasion of epithelial cells by *S. choleraesuis* and *S. typhimurium* were found to require de novo synthesis of several new bacterial proteins. This inducible event appears to be a coordinately regulated system dependent on trypsin- and neuraminidase-sensitive structures present on the epithelial cell surface. Mutants of *S. choleraesuis* and *S. typhimurium* were unable to synthesize these proteins and did not stably adhere to nor invade eukaryotic cells. Two such *S. typhimurium* mutants were avirulent in mice, an indication that these proteins are required for *Salmonella* virulence.**

SALMONELLOSIS IS A MAJOR HEALTH problem throughout the world. In the United States this disease accounts for 40,000 reported cases, 500 deaths, and health care costs exceeding $50 billion annually (*1*). *Salmonella* species are intracellular parasites, and it is thought that these bacteria gain access to their host by penetrating through intestinal epithelial cells (*2*). However, the molecular mechanisms used by these organisms to adhere to, enter, and penetrate through epithelial cells remain poorly defined.

We previously demonstrated that *S. choleraesuis*, a highly invasive *Salmonella* species, could enter, multiply, and proceed through viable MDCK (Madin Darby canine kidney) epithelial cells grown on permeable filters (*3*). Inhibitors of bacterial RNA or protein synthesis blocked penetration in this model system. Other workers found that *Salmonella* adherence consists of two phases of attachment—reversible (bacteria can be removed by washing) and irreversible (resistant to washing)—and that addition of chloramphenicol inhibited stable (irreversible) adherence of *S. typhimurium* to small intestinal enterocytes from rats (*4*). These data were consistent with the idea that *Salmonella* penetrate through viable epithelial monolayers impermeable to other bacteria and that *Salmonella* species must synthesize proteins essential to this process. We now report that several bacterial proteins required for *Salmonella* internalization into host cells are induced by epithelial cells.

The kinetics of stable *S. choleraesuis* adherence and invasion of MDCK cells are presented in Fig. 1. Few bacteria adhered to MDCK monolayers within the first 2 hours, but binding then increased exponentially,

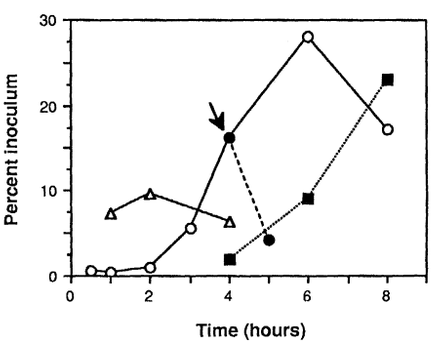

Fig. 1. Kinetics of *S. choleraesuis* adherence and invasion to MDCK epithelial cells. The percentage of bacteria adherent to glutaraldehyde-fixed monolayers (○) and the percentage of bacteria that invaded viable monolayers (■) were determined as described in Table 1. The effect of adding chloramphenicol or rifampin or cooling to 4°C for 1 hour after bacteria have bound to fixed monolayers for 4 hours is illustrated (●). The arrow indicates when these agents were applied. The percentage of bacteria bound to new fixed monolayers after incubation for 3 hours with a fixed monolayer followed by vigorous pipetting before incubation with the new fixed monolayer is shown (△). Values are an average of duplicate samples and are representative data from one of three experiments.

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