INFECTION AND IMMUNITY, May 1995, p. 1820–1826 Vol. 63, No. 5
0019-9567/95/$04.00+0

Regulation of Macrophage Activation and Human Immunodeficiency Virus Production by Invasive *Salmonella* Strains

STEVEN B. MIZEL,* LOUIS S. KUCERA, STEPHEN H. RICHARDSON, FEDERICA CIACCI, AND NATHAN P. IYER

Department of Microbiology and Immunology, Wake Forest University Medical Center, Winston-Salem, North Carolina 27157

Received 15 November 1994/Returned for modification 10 February 1995/Accepted 22 February 1995

Salmonellae possess the ability to adhere to and invade macrophages and in so doing trigger a number of intracellular events that are associated with cellular activation. As an initial approach to defining the mechanisms by which invasive salmonellae alter macrophage function, we have explored the impact of *Salmonella* infection on the production of human immunodeficiency virus (HIV) in U1 cells, a promonocytic cell line latently infected with the virus. Infection of U1 cells with a pathogenic strain of *Salmonella enteritidis* resulted in a marked induction of macrophage activation and HIV production. The stimulatory effect of salmonellae was mediated by signals other than lipopolysaccharide. *Salmonella* mutants with specific defects in invasion or intracellular survival were markedly less effective in the induction of HIV production. In contrast to *S. enteritidis*, strains of *Yersinia enterocolitica*, *Legionella pneumophila*, and *Escherichia coli* did not induce HIV production. However, all of these bacteria induced comparable levels of gene expression mediated by the HIV long terminal repeat. The results of this study are consistent with the notion that invasive salmonellae possess the ability to activate the macrophage by at least one mechanism that is not shared with several other species of gram-negative bacteria. Furthermore, the expression of this unique property is maximal with *Salmonella* strains that are not only invasive but also capable of prolonged survival within the macrophage. Our results indicate that the U1 cell line may be a very useful model system with which to examine the biochemical pathways by which internalized salmonellae modulate the activation state of the macrophage.

Salmonellae are facultative intracellular pathogens that cause a spectrum of diseases ranging from gastroenteritis to severe systemic infections such as typhoid fever. The *Salmonella enteritidis* serotypes that cause gastroenteritis invade the intestinal mucosa and are found primarily within mucosal epithelial cells and tissue-fixed macrophages. Although the majority of internalized salmonellae are destroyed, the available evidence indicates that invasive salmonellae can persist in phagosomes for various periods (2) and modify host cell behavior (1, 2, 7, 10, 43, 48). More than a dozen distinct loci have been implicated in the control of *Salmonella* invasion (9, 18, 20, 24, 28, 30, 43, 50) and intracellular survival (7, 8, 14, 20, 21, 35, 38–41). During the attachment and invasion process, salmonellae trigger an increase in several host cell second messengers that may be required for entry, including an elevation in intracellular calcium, phospholipase A_2 activity, and leukotriene production (43), as well as enhanced protein kinase activity (43, 48). These second messengers may play important roles in the functional activation of macrophages induced by a broad spectrum of extracellular signals such as cytokines, bacterial cell wall components, and antigen-antibody complexes (11, 16, 33, 42, 51). However, very little is known about the intracellular interactions that may occur between internalized salmonellae and the host macrophage.

In addition to its susceptibility to infection by invasive *Salmonella* strains, the macrophage is also a target for infection by human immunodeficiency virus (HIV) (27, 34). It is well established that the macrophage is a site for HIV production and a major reservoir for the production and spread of HIV in the infected host (26, 27, 34, 53). Folks et al. (22) developed a macrophage cell line model, termed U1, that is latently infected with HIV, producing only very low levels of virus in the absence of external stimuli. As with normal macrophages, HIV production in U1 cells is markedly enhanced by factors that promote cellular activation. For example, the cytokines granulocyte-macrophage colony-stimulating factor (22, 45) and tumor necrosis factor alpha (37), as well as tetradecanoyl phorbol-13-acetate (TPA) (45), activate HIV production in U1 cells. In view of the ability of salmonellae to stimulate pathways associated with the activation state of macrophages (43, 48) and the importance of cellular activation on the production of HIV in macrophages (27), we examined the effect of salmonellae on the production of HIV in U1 cells.

MATERIALS AND METHODS

Cells. U1 cells, a subclone of the promonocytic cell line U937 latently infected with HIV (22), were used as a target for invasion by *Salmonella* strains. The cells were routinely cultured in RPMI 1640 containing 20% fetal bovine serum (FBS; Gibco-Life Technologies). U38 cells, a subclone of U937 cells stably transfected with the HIV long terminal repeat (LTR) promoter linked to the chloramphenicol acetyltransferase (CAT) gene (19), were obtained from the NIH AIDS Research and Reference Reagent Program, and U937 cells were obtained from the American Type Culture Collection. U937 and U38 cells were grown in RPMI 1640 containing 10% FBS and 50 μg of gentamicin per ml.

Bacteria. *S. enteritidis* CDC5str (CDC), a clinical isolate from a case of gastroenteritis (50), *S. enteritidis* SM5T (SM), an isogenic Tn*phoA* insertion mutant of the CDC strain with a mutation in the *pagC* gene that results in invasion and macrophage survival defects (41), *Yersinia enterocolitica* 8081V, a serotype O:8 isolate from a fatal septicemia, and *Y. enterocolitica* 8081C, a plasmid-cured derivative of strain 8081V (46), were provided by Virginia Miller. *Salmonella typhimurium* SB111, a mutant defective for invasion of host cells (43), was obtained from Jorge Galan. *Legionella pneumophila* AA100, AA108, and AA109, isogenic mutants that differ in expression of macrophage infectivity potentiator, a factor required for normal intracellular growth (29), were provided by Cary Engleberg. *Escherichia coli* DH5α was purchased from Gibco-Life Technologies, and *E. coli* HB101 was purchased from Invitrogen. *E. coli* MM294 was kindly provided by Ian Blomfield. All test strains were subcultured once on solid media

* Corresponding author. Mailing address: Department of Microbiology and Immunology, Wake Forest University Medical Center, Medical Center Blvd., Winston-Salem, NC 27157. Phone: (910) 716-4471. Fax: (910) 716-9928.

from the original stocks that were stored at −70°C. Subcultures were resuspended in heart infusion broth (Difco) supplemented with 50% (vol/vol) sterile glycerol and stored at −70°C in 96-well microtiter plates. Prior to each experiment, the contents of a single well were transferred to 5 ml of heart infusion broth or, in the case of *L. pneumophila*, to plates of buffered charcoal-yeast extract agar. Salmonellae were grown overnight on a roller apparatus at 37°C, yersiniae were grown in 50-ml flasks on a reciprocal shaker at 30°C, and legionellae were grown at 37°C in a humidified atmosphere of 5% CO_2. Cell densities were determined turbidimetrically. Bacteria were in the mid- to late exponential phase of growth at the time of use.

Bacterial infection of the U1 promonocytic-like cell line. The required number of bacteria were pelleted in microcentrifuge tubes, washed once with phosphate-buffered saline (PBS), and added to tubes containing U1 cells. The cells were obtained from log-phase cultures and were washed twice with ice cold RPMI 1640 medium prior to infection with bacteria. Preliminary experiments revealed that the adherence and invasiveness of the bacteria were maximal after approximately 2 h at 37°C. Therefore, in subsequent experiments, U1 cells were incubated with the bacteria at a multiplicity of infection (MOI) of 1, 10, or 100 for 2 h at 37°C with frequent mixing, washed twice with ice-cold RPMI 1640 medium (without FBS), and resuspended at 5×10^5 cells per ml in RPMI 1640 containing 20% FBS and 100 μg of gentamicin per ml. Gentamicin sensitivity was used to distinguish between extracellular and intracellular bacteria at later times, since the antibiotic does not enter cells (31). Maximal killing of the bacteria in the presence of gentamicin is achieved after approximately 60 min (data not shown). The number of cell-associated bacteria was determined by removing 100-μl aliquots of the cells after infection, washing the cell pellets twice with PBS, lysing the cell pellets in 4 ml of 0.1% Triton X-100, and plating dilutions on agar medium.

To estimate the distribution of cell surface-bound versus intracellular bacteria, bacteria were stained with the nontoxic lipophilic vital stain PKH-2 (Sigma Immunochemicals) as described by Raybourne and Bunning (47). This treatment does not alter the replication, binding, or internalization of the bacteria. At appropriate intervals, the bacterium-promonocytic cell complexes were washed with PBS, fixed with fresh 2% paraformaldehyde in PBS for 2 to 4 h at 4°C, washed and resuspended in PBS containing 0.5% bovine serum albumin, 0.1% glycine, and 0.02% sodium azide, and stored at 4°C. The fluorescence of extracellular bacteria was quenched by staining with crystal violet or by including antibody or 10% FBS in the mounting medium. Aliquots of the fixed-stained mixtures were suspended in glycerol-based mounting medium under coverslips and examined by fluorescence microscopy.

Reverse transcriptase assay. Reverse transcriptase activity was used as a measure of HIV production (32). Briefly, 1 ml of cell suspension was precipitated overnight at 4°C with 40 μl of 4 M NaCl and 480 μl of 30% (wt/vol) Carbowax polyethylene glycol 8000. The resulting precipitate was collected following centrifugation and resuspended in 60 μl of a 2:1 solution of buffer A (25 mM Tris-HCl [pH 7.5], 50 mM KCl, 0.025% Triton X-100, 50% glycerol, 5 mM dithiothreitol) and buffer B (1.5 M KCl, 0.9% Triton X-100). The resuspended precipitate was incubated on ice for 5 min. Aliquots (2 μl) were added to 100 μl of reaction mixture containing 80 μl of template buffer (40 mM Tris-HCl [pH 7.5], 40 mM KCl, 5 mM dithiothreitol, 10 mM EGTA, 3 μl of [^{3}H]dTTP [16 Ci/mmol; NEN DuPont]), 0.5 μg of poly(rA) · poly$(dT)_{12-18}$ primer (10 μl), and 8 μl of 2% $MgCl_2$ and incubated for 1 h at 37°C. The reactions were stopped by the addition of 1 ml of 20% (wt/vol) trichloroacetic acid. After 2 h on ice, the precipitates were collected on filters and washed three times with ice-cold 5% trichloroacetic acid and once with 95% ethanol. The dried filters were placed in scintillation vials, and the radioactivity was measured by a liquid scintillation counter.

Transfection of macrophages and assay of CAT activity. U38 cells were transiently transfected by the DEAE-dextran method as previously described (13). The pSV2tat72 vector containing a synthetic gene encoding the first 72 amino acids of the Tat transactivating protein under the transcriptional control of the simian virus 40 early promoter (23) was obtained from the NIH AIDS Research and Reference Reagent Program. Following transfection, the U38 cells were incubated for 22 to 24 h prior to infection with bacteria. Infected and control cells were harvested after an additional 24 h, and the level of CAT activity in cell lysates was measured as previously described (13).

RESULTS

Effect of salmonellae on HIV production. To assess the effect of the CDC strain of *S. enteritidis* on HIV production in U1 cells, the U1 cells were incubated with the bacteria at an MOI of 10 for 2 h at 37°C, washed, and incubated for 72 h (in medium containing gentamicin to kill extracellular bacteria) prior to the measurement of total (cells plus medium) reverse transcriptase activity. As controls, U1 cells were independently incubated without stimulation or with TPA, a potent inducer of HIV production in U1 cells (45). As shown in Fig. 1, unstimulated U1 cells exhibited little if any viral production (as detected by reverse transcriptase activity), whereas TPA induced a substantial increase in HIV production. The CDC strain also markedly stimulated an increase in HIV production. Qualitatively similar data were obtained when cell lysates and culture medium were independently tested for reverse transcriptase activity (data not shown). The level of CDC-induced HIV production was reduced when an MOI of 1 or 100 was used (see Fig. 4 for results at an MOI of 100), and thus a CDC MOI of 10 was used in all subsequent experiments.

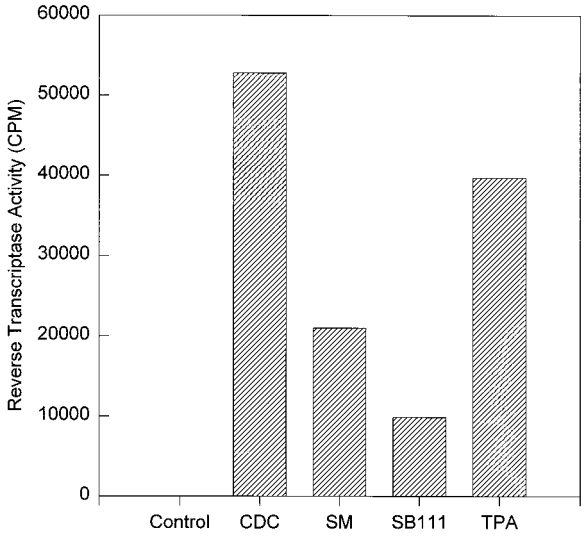

FIG. 1. Induction of HIV production in U1 cells by *Salmonella* strains. Log-phase U1 cells (10^6) were incubated at an MOI of 10 with bacteria suspended in 1 ml of RPMI 1640 medium for 2 h at 37°C with frequent mixing. The cells were then pelleted, washed twice with serum-free RPMI medium, and resuspended at a density of 5×10^5 cells per ml in RPMI 1640 medium containing 20% FBS and 100 μg of gentamicin per ml. Alternatively, U1 cells were left untreated or stimulated with 10 nM TPA. Cells and culture media were harvested after 72 h and kept frozen until assayed for reverse transcriptase activity (32). The results are representative of more than 10 separate experiments.

To assess the possible contributions of bacterial adherence, invasiveness, and intracellular survival to the overall effect of salmonellae on HIV production, we also tested the effect of *Salmonella* strains SM and SB111. Although HIV production was stimulated by the SM and SB111 strains, these strains were less effective than the CDC strain (Fig. 1). Analysis of the data from 10 separate experiments revealed that the levels of reverse transcriptase activity induced with the SM and SB111 strains at an MOI of 10 were approximately 46% ± 26% and 18% ± 20% of that achieved with the CDC strain. However, it should be noted that the SM strain occasionally induced a level of HIV production that was similar to that achieved with the CDC strain (88% of the CDC level in 1 of 10 experiments and 73 and 79% in 2 of 10 experiments). The results of these experiments are consistent with the notion that invasion and intracellular survival play important roles in the effect of salmonellae on HIV production.

To ensure that the more limited effect of the SM and SB111 strains of HIV production was indeed due to differences in invasiveness (SB111) or intracellular survival (SM), we evaluated the adherence, invasion, and intracellular survival of these two strains and compared them with the corresponding properties of the CDC strain. Incubation of U1 cells with the CDC, SM, and SB111 strains resulted in the adherence of relatively equivalent numbers of organisms (Fig. 2). In the experiment presented in Fig. 2, 2 to 4% of the input CDC, SM, and SB111 cells were associated with the U1 cells after an attachment

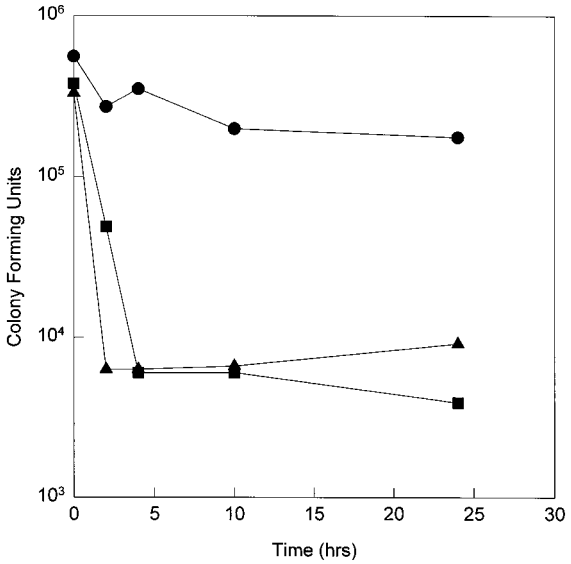

FIG. 2. Adherence and intracellular survival of *Salmonella* strains CDC, SM, and SB111. U1 cells were incubated for 2 h at 37°C with each bacterial strain at an MOI of 20 and then washed to remove unbound bacteria. Aliquots of the U1 cells were then lysed with an equal volume of 0.5% Triton X-100, vortexed, and plated at appropriate dilutions. Following the removal of the initial samples, the U1 cultures were supplemented with 100 μg of gentamicin per ml. Since extracellular but not intracellular bacteria are killed by gentamicin, it is possible to measure the number of intracellular bacteria by adding gentamicin and plating Triton X-100 lysates of the macrophages on Columbia agar (31). The treated U1 cells were washed to remove gentamicin prior to lysing with Triton X-100. Maximal killing of the bacteria in the presence of gentamicin is achieved after approximately 60 min. ●, CDC; ■, SM; ▲, SB111.

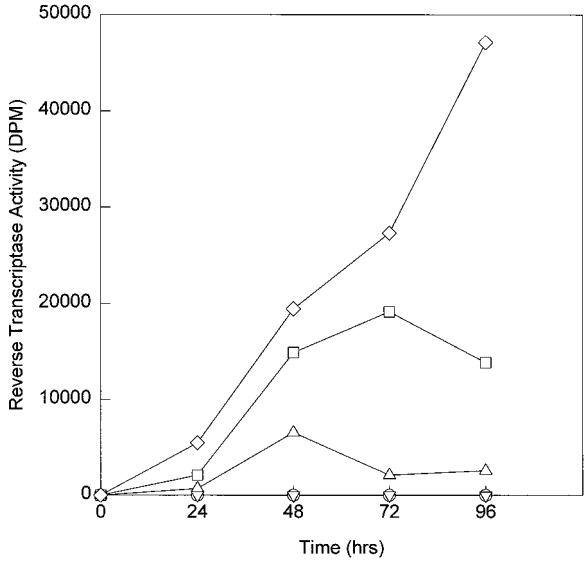

FIG. 3. Time course for the induction of HIV production in U1 cells by *Salmonella* strains. The conditions of the experiment were as for Fig. 1. ○, control; □, CDC; △, SM; ▽, SB111; ◇, TPA-treated cells. The results are representative of three separate experiments.

period of 2 h at 37°C. During a 24-h period following the initial exposure to the bacteria, the number of intracellular CDC cells gradually declined to a level that was approximately 31% of that obtained after removal of the unbound bacteria at the end of the initial 2-h incubation period. In contrast to the results with the CDC strain, the SM strain displayed a more rapid rate of disappearance, with approximately 10% of the cell-associated bacteria surviving 2 h after the addition of gentamicin. By 24 h, less than 1% of the initial level of cell-associated SM bacteria was present. As would be expected (43), the weakly invasive SB111 strain exhibited a very rapid rate of decline since these bacteria were, in large part, extracellular and thus highly susceptible to killing by the added gentamicin. Thus, the effectiveness of the CDC, SM, and SB111 strains of *Salmonella* as inducers of HIV production in U1 cells correlates with the invasive and survival properties of each strain.

When U937 cells, the uninfected parent cell line of the U1 cells, were incubated with the CDC strain (stained with the nontoxic lipophilic dye PKH-2 [47]) at an MOI of 10, 5 to 10% of the U937 cells contained intracellular bacteria following a 2-h exposure. It is important to note that the TPA response (Fig. 1) is most likely a measure of most if not all of the U1 cells, whereas the results obtained with the CDC strain reflect an initial response involving only a small percentage of the total cell population (i.e., the 5 to 10% of the cells that had bound and internalized the bacteria). Although the strong response to salmonellae may be limited to the cells initially infected by the bacteria, it is also possible that the overall response involves the recruitment of neighboring cells that were not invaded by the bacteria, perhaps via the release of a soluble factor(s) or specific cell-cell interactions.

The time course for the induction of reverse transcriptase activity by salmonellae is presented in Fig. 3. An increase in reverse transcriptase activity was detected within 24 h after addition of the bacteria and peaked between 48 to 72 h postinfection. The reduced ability of the SM and SB111 strains to induce HIV production was evident throughout the 72-h incubation. In contrast to the leveling off of activity in response to the bacteria, TPA induced a prolonged increase in the induction of reverse transcriptase activity. The difference in kinetics between TPA-treated and CDC-infected U1 cells may be due to the declining survival of the CDC cells during the period following invasion (Fig. 2).

Lack of an effect of LPS on HIV production in U1 cells. Several lines of evidence indicate that the effect of salmonellae on HIV production is mediated by a mechanism that does not involve lipopolysaccharide (LPS), a component of the outer membrane of gram-negative bacteria and a well-known macrophage stimulant. First, the results with the CDC, SM, and SB111 strains indicate that the mere presence of LPS is not a sufficient trigger for induction of maximal HIV production. In confirmation of previous results (5, 44), LPS (0.001 to 10 μg/ml) had no effect on HIV production in U1 cells (data not shown). Furthermore, three other gram-negative strains did not induce HIV production in U1 cells (Table 1 and Fig. 4). As shown in Table 1, neither *E. coli* DH5α, *Y. enterocolitica*, nor *L. pneumophila* stimulated HIV production in U1 cells at an MOI of 10. Furthermore, these bacterial strains were equally ineffective at MOIs of up to 100 (Fig. 4), indicating that the inability to induce HIV production was not simply due to a difference in the numbers of bacteria required to elicit a response. Thus, the ability to stimulate HIV production in the latently infected U1 cell line may be due to a novel set of interactions between internalized salmonellae and the host cell.

Effect of various bacterial strains on HIV LTR-mediated gene expression. Once HIV DNA has been integrated into host DNA, it serves as the template for viral RNA synthesis, a process that is regulated by host cell and viral transcription factors (for reviews, see references 4 and 15). LTRs are present

TABLE 1. Effects of different bacterial strains on HIV production in U1 cells

Addition[a]	Reverse transcriptase activity (dpm)[b]
None	2,746
TPA	24,424
S. enteritidis CDC	36,366
S. enteritidis SM	16,203
S. typhimurium SB111	10,983
Y. enterocolitica 8081C	3,401
Y. enterocolitica 8081V	4,186
E. coli DH5α	5,109
L. pneumophila AA100	2,229
L. pneumophila AA108	2,724
L. pneumophila AA109	1,546

[a] Bacteria were used at an MOI of 10.
[b] The results are representative of five separate experiments.

at both ends of the viral genome and contain many of the critical *cis*-acting sequences that are specifically recognized by the host cell and viral transcription factors that play essential roles in the initiation and propagation of viral transcription. In view of the critical role that the viral LTR plays in HIV production, we examined the effect of salmonellae on the activity of the HIV-LTR in U38, a clone of U937 cells that is stably transfected with a construct containing the CAT gene under the transcriptional control of the HIV LTR (19). In addition to testing the singular effect of the bacteria, we also evaluated the impact of the expression of the viral regulatory protein, Tat, on HIV LTR activity. The available evidence indicates that Tat may enhance transcription driven by the HIV LTR by two mechanisms, namely, by promoting transcriptional elongation and possibly by stimulating transcriptional initiation (4, 15). Three representative experiments are shown in Fig. 5. Although the extent of stimulation was somewhat variable from experiment to experiment, the data from three representative

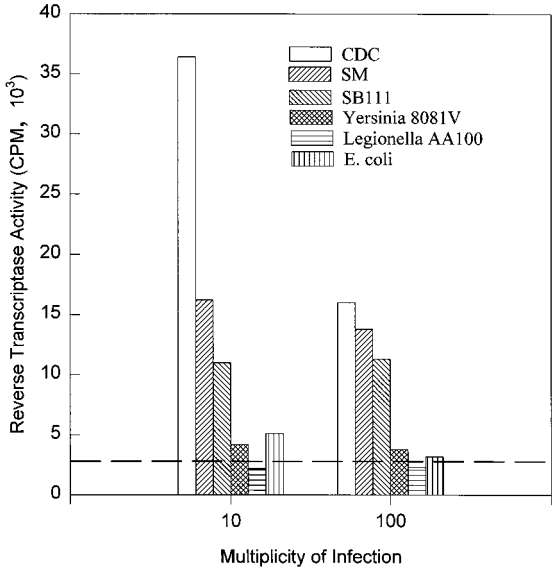

FIG. 4. Effects of bacterial strains at different MOIs on HIV production in U1 cells. The various bacterial strains were obtained at mid- to late exponential growth and were used at an MOI of 10 or 100 to infect U1 cells. Reverse transcriptase activity was measured after 72 h. The level of reverse transcriptase activity in cultures of unstimulated U1 cells is shown by the broken line.

experiments show that incubation of U38 cells with the CDC strain of *Salmonella* (in the absence of Tat) results in a significant increase in CAT induction. However, the magnitude of the response to salmonellae was dramatically enhanced when the U38 cells were transiently transfected with an expression plasmid encoding the HIV Tat protein (pSV2tat72) prior to *Salmonella* infection. This is particularly evident at low concentrations of pSV2tat72 (i.e., 1 μg). Generally, at high concentrations of pSV2tat72, comparable levels of CAT activity were observed with cells incubated in the presence or absence of salmonellae. These results indicate that salmonellae possess the ability to stimulate the production or activation of necessary host cell transcription factors that in the absence of viral Tat are generally present at sufficient levels to trigger HIV LTR-mediated gene expression. Tat expression results in a synergistic response, as evidenced by the high level of CAT activity in cultures transfected with the Tat expression plasmid and infected with the CDC strain of *Salmonella*. We have found that the synergism between salmonellae and Tat is evident with concentrations of pSV2tat72 as low as 10 ng. Although we have consistently found that salmonellae synergize with low levels of Tat expression, we have noted qualitative differences in the results. For example, in some experiments, the effect of salmonellae or Tat was evident only in the presence of both stimuli (for example, Fig. 5B). It is likely, therefore, that comparable levels of HIV LTR-mediated gene expression may be achieved at low levels of host transcription factors and high Tat expression or high levels of host transcription factors and low or minimal Tat expression. The sensitivity of the host cell to activation by salmonellae or Tat may be dependent on slight variations in the intracellular environment prior to bacterial infection or, in the case of salmonellae, to small but important variations in the physiological state of the bacteria used in each experiment.

Since the *Salmonella* mutant strains SB111 and SM exhibit a reduced ability to induce HIV production (Fig. 1 and 4), we examined the abilities of these strains, as well as strains of *Y. enterocolitica* and *E. coli*, to activate gene expression under the control of the HIV LTR. In view of the generally accepted notion that many agents induce HIV production by activating the HIV LTR (4, 15), our initial assumption was that the strains that did not induce HIV production would have little if any effect on the HIV LTR. However, as shown in Fig. 6, *Salmonella* strains SB111 and SM, as well as *E. coli* MM294 and *Y. enterocolitica* 8081V, activated significant HIV-LTR-mediated gene expression in U38 cells. A summary of the results of several experiments is presented in Table 2. The SM strain was as effective as the CDC strain, whereas the SB111 strain induced a level of HIV LTR-mediated gene expression in U38 cells that was approximately 70% of that obtained with the CDC strain. Both strains of *E. coli* and *Y. enterocolitica* 8081V were slightly less effective than *Salmonella* strain SB111, inducing levels of HIV LTR-mediated gene expression that were 50 to 60% of that obtained with the CDC strain. The activation of the HIV LTR by all of the bacterial strains was not mediated via LPS, as evidenced by the observation that LPS itself had no effect on the activation of HIV LTR-mediated gene expression (data not shown). Finally, all of the bacterial strains induced comparable levels of HIV LTR-mediated gene expression in U38 cells transiently transfected with the Tat expression plasmid (data not shown).

These results indicate that the stimulatory effect of the CDC strain of *Salmonella* on HIV production is not mediated solely via activation of the HIV LTR. In addition to its stimulatory effect on the HIV LTR, the CDC strain, in contrast to the other bacterial strains, must have a stimulatory effect, either

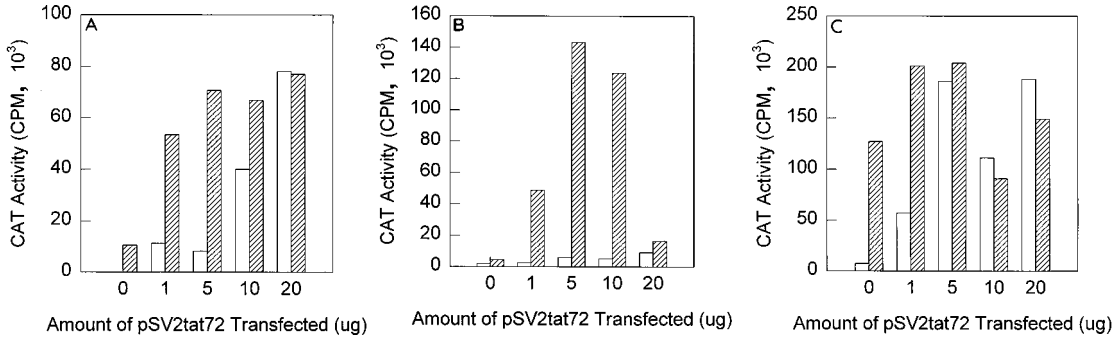

FIG. 5. Effect of *Salmonella* strain CDC on the activation of HIV LTR-mediated gene expression in U38 cells. Cultures of U38 cells stably expressing a CAT gene under the control of the HIV LTR were left untreated for 24 h prior to infection with bacteria or were transiently transfected with increasing amounts of the Tat expression plasmid pSV2tat72 and then incubated for 24 h. Subsequently, the cultures were infected with the CDC strain for 2 h at 37°C and then incubated for an additional 24 h prior to the assay of CAT activity. Results of three independent experiments are presented. □, control; ▨, CDC.

directly or indirectly, on one or more viral regulatory components involved in HIV production.

DISCUSSION

Our results demonstrate that a pathogenic strain of *Salmonella*, in contrast to strains of *Y. enterocolitica*, *L. pneumophila*, and *E. coli*, possesses the ability to dramatically enhance HIV production in a promonocytic cell line latently infected with the virus. In addition, our findings raise the possibility that secondary *Salmonella* infections may have an impact on the progression of HIV infection. By weakening the host immune system, HIV may predispose an individual to infection by the gastrointestinal pathogen *S. enteritidis*, an event that may in turn promote the production of the virus in mucosal macrophages and perhaps other susceptible mucosal cell types. The observation that macrophage-tropic HIV strains are present in bowel tissue (6, 34) is consistent with this hypothesis. The existence of such a feedback amplification loop might accelerate the course of the disease process and thus the development of AIDS in individuals who experience both types of infections.

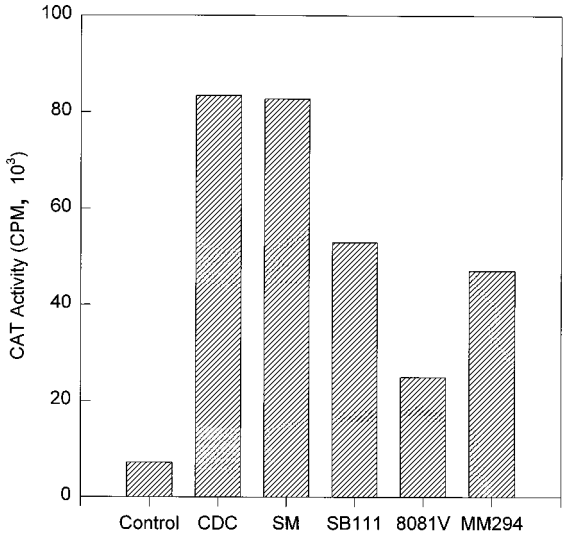

FIG. 6. Effects of *Salmonella*, *E. coli*, and *Yersinia* strains on the activation of HIV LTR-mediated gene expression in U38 cells. The U38 cells were treated as described in the legend to Fig. 4. Equivalent numbers of bacteria were used for each infection. The results are representative of several experiments.

In view of the very limited ability of the noninvasive SB111 strain of *Salmonella* to induce HIV production, it is evident that a significant component of the stimulatory signal must arise as a consequence of bacterial invasion of the macrophage. Furthermore, the reduced effectiveness of the SM strain indicates that the level of HIV production may be related to the survival time of the salmonellae within the U1 cell. However, the possibility remains that the process of invasion itself contributes substantially to the activation events that lead to HIV production, possibly by triggering changes in the intracellular level of calcium or lipid mediators or mitogen-activated protein kinase activity (43). Although intracellular survival appears to enhance the overall effectiveness of salmonellae in macrophage activation and HIV production, it is important to emphasize that intracellular survival by itself is apparently not sufficient for induction of HIV production. For example, *Y. enterocolitica* 8081C and 8081V exhibit levels of invasion and intracellular survival in U1 cells that are equal to if not greater than those achieved with the *Salmonella* strains used in this study (unpublished observations). Thus, *Salmonella* strains, as opposed to *Y. enterocolitica*, *E. coli*, or *L. pneumophila*, possess a novel ability to enhance the activity of a pathway(s) that is centrally involved in the processes of macrophage activation and viral production. Following the preparation of this report, we became aware of a recent study by Andreana et al. (3) demonstrating the induction of HIV by *S. typhimurium* in U1 cells. Although these investigators also found that a *Salmonella* strain enhances HIV production, they did not explore the effect of other bacterial species, the re-

TABLE 2. Relative effectiveness of various bacterial strains as inducers of HIV LTR-mediated gene expression

Bacterial strain	Effectiveness as an inducer of HIV LTR-mediated gene expression[a] (mean ± SD)	n[b]
S. enteritidis SM	1.04 ± 0.35	8
S. typhimurium SB111	0.69 ± 0.18	8
Y. enterocolitica 8081V	0.46 ± 0.30	8
E. coli HB101	0.53 ± 0.33	5
E. coli MM294	0.62 ± 0.30	3

[a] Relative to the effectiveness of *S. enteritidis* CDC.
[b] n = number of experiments.

quirement for *Salmonella* invasion and intracellular survival, or the possibility that *Salmonella* strains may use more than one stimulatory mechanism to promote HIV production.

The results of our experiments on the activation of HIV LTR gene expression (Fig. 6 and Table 2) indicate that the induction of HIV production by salmonellae is not simply the result of the activation of the HIV LTR, since bacterial strains that do not induce HIV production in U1 cells were effective inducers of HIV LTR-mediated gene expression. Pomerantz et al. (45) initially suggested that the latency state of U1 cells might be due to a low-level synthesis of Rev, an HIV-derived regulatory factor that plays a pivotal role in the HIV life cycle by regulating the switch from the early phase to the late phase of HIV infection during which genomic RNA as well as the Gag and Gag-Pol mRNAs begin to appear (4, 15, 52). Rev may induce this switch by enhancing the transport, splicing, or translation of RNAs containing a Rev response element (4, 15, 52). Duan et al. (17) have reported that HIV production in U1 cells can be enhanced if the cells are induced to express Tat encoded by a retroviral shuttle vector. In contrast, Rev expression resulted in only a modest increase in virus production. Cannon et al. (12) have also found that Tat expression or the addition of exogenous Tat can enhance virus production in U1 cells, perhaps by increasing viral RNA expression above a critical level. It is possible, therefore, that salmonellae may promote viral production by acting in a generic manner to stimulate the HIV LTR (Fig. 6) via the induction of factors such as NF-κB and Sp1 and in a *Salmonella*-specific manner by enhancing the activity of Rev, perhaps by influencing important interactions between Rev and other proteins (4). In addition, invasive salmonellae may also stimulate the activity of Tat and in so doing promote the synthesis of high levels of Rev. Studies are in progress to evaluate these possibilities.

In addition to defining the ultimate effector of *Salmonella* action, it will be important to determine the signaling pathway(s) that links these phagosome-enclosed organisms with the host cell intracellular environment. Our findings are consistent with the notion that invasive salmonellae may possess the ability to alter the intracellular environment of the macrophage by a mechanism(s) that is not shared with several other gram-negative bacteria (*E. coli*, *Y. enterocolitica*, or *L. pneumophila*). The results of a number of recent studies (see, for example, references 13, 36, 49, and 54) indicate that protein kinases and protein phosphatases control the DNA-binding and transactivating activities of transcription factors such as NF-κB, CREB, and AP-1 that are associated with gene expression in activated macrophages. The observation that salmonellae can stimulate host cell protein phosphorylation (43, 48) may indicate that these organisms produce and secrete a protein kinase or, alternatively, an activator of a host cell protein kinase that may positively regulate the activity of one or more of these transcription factors. In this regard, it is worth noting that the production and secretion of a serine/threonine protein kinase appears to be an important virulence determinant of *Yersinia pseudotuberculosis* (25).

In conclusion, the results presented in this study indicate that the U1 cell may be a very useful model system with which to probe the intracellular events by which invasive salmonellae modulate the behavior of macrophages. Furthermore, the U1 cell system may also prove to be of substantial value in defining bacterial genetic loci that may be associated with the relatively selective action of salmonellae on macrophage activation and HIV production.

ACKNOWLEDGMENTS

We thank V. Miller for the *Salmonella* and *Yersinia* strains, Jorge Galan for *Salmonella* strain SB111, and C. Engleberg for the *Legionella* strains.

This work was supported in part by Public Health Service grants AI27163 (to S.B.M.) and CA12197 (Comprehensive Cancer Center of Wake Forest University).

REFERENCES

1. **Abshire, K. Z., and F. C. Neidhardt.** 1993. Growth rate paradox of *Salmonella typhimurium* within host macrophages. J. Bacteriol. **175:**3744–3748.
2. **Alpuche-Aranda, C. M., E. L. Racoosin, J. A. Swanson, and S. I. Miller.** 1994. *Salmonella* stimulate macrophage macropinocytosis and persist within spacious phagosomes. J. Exp. Med. **179:**601–608.
3. **Andreana, A., S. Gollapudi, C. H. Kim, and S. Gupta.** 1994. *Salmonella typhimurium* activates human immunodeficiency virus type I in chronically infected promonocytic cells by inducing tumor necrosis factor-α production. Biochem. Biophys. Res. Commun. **201:**16–23.
4. **Antoni, B. A., S. B. Stein, and A. B. Rabson.** 1994. Regulation of human immunodeficiency virus infection: implications for pathogenesis. Adv. Virus Res. **43:**53–145.
5. **Bagasra, O., S. D. Wright, T. Seshamma, J. W. Oakes, and R. J. Pomerantz.** 1992. CD14 is involved in control of human immunodeficiency virus type 1 expression in latently infected cells by lipopolysaccharide. Proc. Natl. Acad. Sci. USA **89:**6285–6289.
6. **Barnett, S., A. Barboza, C. M. Wilcox, C. E. Forsmark, and J. A. Levy.** 1991. Characterization of human immunodeficiency virus type 1 strains recovered from the bowel of infected individuals. Virology **182:**802–809.
7. **Baumler, A. J., J. G. Kusters, I. Stojiljkovic, and F. Heffron.** 1994. *Salmonella typhimurium* loci involved in survival within macrophages. Infect. Immun. **62:**1623–1630.
8. **Behlau, I., and S. I. Miller.** 1993. A PhoP-repressed gene promotes *Salmonella typhimurium* invasion of epithelial cells. J. Bacteriol. **175:**4475–4484.
9. **Bliska, J. B., J. E. Galan, and S. Falkow.** 1993. Signal transduction in the mammalian cell during bacterial attachment and entry. Cell **73:**903–920.
10. **Buchmeier, N. A., and F. Heffron.** 1989. Intracellular survival of wild-type *Salmonella typhimurium* and macrophage-sensitive mutants in diverse populations of macrophages. Infect. Immun. **57:**1–7.
11. **Buchmuller-Rouiller, Y., and J. Mauel.** 1991. Macrophage activation for intracellular killing as induced by calcium ionophore. Correlation with biologic and biochemical events. J. Immunol. **146:**217–223.
12. **Cannon, P., S.-H. Kim, C. Ulich, and S. Kim.** 1994. Analysis of Tat function in human immunodeficiency virus type 1-infected low-level-expression cell lines U1 and ACH-2. J. Virol. **68:**1993–1997.
13. **Chedid, M., B. K. Yoza, J. W. Brooks, and S. B. Mizel.** 1991. Activation of AP-1 by IL 1 and phorbol esters in T cells: role of protein kinase A and protein phosphatases. J. Immunol. **147:**867–873.
14. **Christman, M. F., R. W. Morgan, F. S. Jacobson, and B. N. Ames.** 1985. Positive control of a regulon for defenses against oxidative stress and some heat-shock proteins in Salmonella typhimurium. Cell **41:**753–762.
15. **Cullen, B. R.** 1991. Regulation of human immunodeficiency virus replication. Annu. Rev. Microbiol. **45:**219–250.
16. **Dong, Z., X. Qi, and I. J. Fidler.** 1993. Tyrosine phosphorylation of mitogen-activated protein kinases is necessary for activation of murine macrophages by natural and synthetic bacterial products. J. Exp. Med. **177:**1071–1077.
17. **Duan, L., J. W. Oakes, A. Ferraro, O. Bagasra, and R. J. Pomerantz.** 1994. Tat and Rev differentially affect restricted replication of human immunodeficiency virus type I in various cells. Virology **199:**474–478.
18. **Elsinghorst, E. A., L. S. Baron, and D. J. Kopecko.** 1989. Penetration of human intestinal cells by *Salmonella*: molecular cloning and expression of *Salmonella typhi* invasion determinants in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **86:**5173–5177.
19. **Felber, B. K., and G. N. Pavlakis.** 1988. A quantitative assay for HIV-1 based on trans-activation. Science **239:**184–187.
20. **Fields, P. I., E. A. Groisman, and F. Heffron.** 1989. A *Salmonella* locus that controls resistance to microbicidal proteins from phagocytic cells. Science **243:**1059–1062.
21. **Fields, P. I., R. V. Swanson, C. G. Haidaris, and F. Heffron.** 1986. Mutants of Salmonella typhimurium that cannot survive within the macrophage are avirulent. Proc. Natl. Acad. Sci. USA **83:**5189–5193.
22. **Folks, T., J. Justement, A. Kinter, C. A. Dinarello, and A. S. Fauci.** 1987. Cytokine induced expression of HIV-1 in a chronically-infected promonocyte cell line. Science **238:**800–802.
23. **Frankel, A. D., and C. O. Pabo.** 1988. Cellular uptake of the tat protein from human immunodeficiency virus. Cell **55:**1189–1193.
24. **Galan, J. E., and R. I. Curtiss.** 1989. Cloning and molecular characterization of genes whose products allow *Salmonella typhimurium* to penetrate tissue culture cells. Proc. Natl. Acad. Sci. USA **86:**6383–6387.
25. **Galyov, E. E., S. Hakansson, A. Forsberg, and H. Wolf-Watz.** 1993. A secreted protein kinase of Yersinia pseudotuberculosis is an indispensable virulence determinant. Nature (London) **361:**730–732.

26. **Gartner, S., and M. Popovic.** 1990. Macrophage tropism of HIV-1. AIDS Res. Hum. Retroviruses **6:**1017–1021.
27. **Gendelman, H. E., J. M. Orenstein, L. M. Baca, B. Weiser, H. Burger, D. C. Kalter, and M. S. Meltzer.** 1989. The macrophage in the persistence and pathogenesis of HIV infection. AIDS **3:**475–495.
28. **Groisman, E. A., and H. Ochman.** 1993. Cognate gene clusters govern invasion of host epithelial cells by Salmonella typhimurium and Shigella flexneri. EMBO J. **12:**3779–3787.
29. **Hurley, M. C., K. Balazovich, M. Albano, N. C. Engleberg, and B. I. Eisenstein.** 1993. *Legionella pneumophila* Mip inhibits protein kinase C, p. 69–70. *In* J. M. Barbaree, R. F. Breiman, and A. P. Dufour (ed.), *Legionella*: current status and emerging perspectives. American Society for Microbiology, Washington, D.C.
30. **Jones, B. D., and S. Falkow.** 1994. Identification and characterization of a *Salmonella typhimurium* oxygen-regulated gene required for bacterial internalization. Infect. Immun. **62:**3745–3752.
31. **Jones, B. D., H. F. Paterson, A. Hall, and S. Falkow.** 1993. *Salmonella typhimurium* induces membrane ruffling by a growth factor-receptor-independent mechanism. Proc. Natl. Acad. Sci. USA **90:**10390–10394.
32. **Kucera, L. S., N. Iyer, E. Leake, A. Raben, E. J. Modest, L. W. Daniel, and C. Piantadosi.** 1990. Novel membrane-interactive ether lipid analogs that inhibit infectious HIV-1 production and induce defective virus formation. AIDS Res. Hum. Retroviruses **6:**491–501.
33. **Letari, O., S. Nicosia, C. Chiavaroli, P. Vacher, and W. Schlegel.** 1991. Activation by bacterial lipopolysaccharide causes changes in the cytosolic free calcium concentration in single peritoneal macrophages. J. Immunol. **147:**980–983.
34. **Levy, J. A.** 1993. Pathogenesis of human immunodeficiency virus infection. Microbiol. Rev. **57:**183–289.
35. **Libby, S. J., W. Goebel, A. Ludwig, N. Buchmeier, F. Bowe, F. C. Fang, D. G. Guiney, J. G. Songer, and F. Heffron.** 1994. A cytolysin encoded by Salmonella is required for survival within macrophages. Proc. Natl. Acad. Sci. USA **91:**489–494.
36. **Manak, J. R., N. de Bisschop, R. M. Kris, and R. Prywes.** 1990. Casein kinase II enhances the DNA binding activity of serum response factor. Genes Dev. **4:**955–967.
37. **Mellors, J. W., B. P. Griffith, M. A. Ortiz, M. L. Landry, and J. L. Ryan.** 1991. Tumor necrosis factor-alpha/cachectin enhances human immunodeficiency virus type 1 replication in primary macrophages. J. Infect. Dis. **163:** 78–82.
38. **Miller, S. I.** 1991. PhoP/PhoQ: macrophage-specific modulators of Salmonella virulence. Mol. Microbiol. **5:**2073–2078.
39. **Miller, S. I., A. M. Kukral, and J. J. Mekalanos.** 1989. A two-component regulatory system (phoP/phoQ) controls Salmonella typhimurium virulence. Proc. Natl. Acad. Sci. USA **86:**5054–5058.
40. **Miller, S. I., and J. J. Mekalanos.** 1990. Constitutive expression of the *phoP* regulon attenuates *Salmonella* virulence and survival within macrophages. J. Bacteriol. **172:**2485–2490.
41. **Miller, V. L., K. B. Beer, W. P. Loomis, J. A. Olson, and S. I. Miller.** 1992. An unusual *pagC*::Tn*phoA* mutation leads to an invasion- and virulence-defective phenotype in salmonellae. Infect. Immun. **60:**3763–3770.
42. **Nakano, M., S. Saito, Y. Nakano, H. Yamasu, M. Matsuura, and H. Shinomiya.** 1993. Intracellular protein phosphorylation in murine peritoneal macrophages in response to bacterial lipopolysaccharide (LPS): effects of kinase-inhibitors and LPS-induced tolerance. Immunobiology **187:**272–282.
43. **Pace, J., M. J. Hayman, and J. E. Galan.** 1993. Signal transduction and invasion of epithelial cells by S. typhimurium. Cell **72:**505–514.
44. **Pomerantz, R. J., M. B. Feinberg, D. Trono, and D. Baltimore.** 1990. Lipopolysaccharide is a potent monocyte/macrophage-specific stimulator of human immunodeficiency virus type 1 expression. J. Exp. Med. **172:**253–261.
45. **Pomerantz, R. J., D. Trono, M. B. Feinberg, and D. Baltimore.** 1990. Cells nonproductively infected with HIV-1 exhibit an aberrant pattern of viral RNA expression: a molecular model for latency. Cell **61:**1271–1276.
46. **Portnoy, D. A., S. L. Moseley, and S. Falkow.** 1981. Characterization of plasmids and plasmid-associated determinants of *Yersinia enterocolitica* pathogenesis. Infect. Immun. **31:**775–782.
47. **Raybourne, R. B., and V. K. Bunning.** 1994. Bacterium-host cell interactions at the cellular level: fluorescent labeling of bacteria and analysis of short-term bacterium-phagocyte interaction by flow cytometry. Infect. Immun. **62:**665–672.
48. **Saito, S., H. Shinomiya, and M. Nakano.** 1994. Protein phosphorylation in murine peritoneal macrophages induced by infection with *Salmonella* species. Infect. Immun. **62:**1551–1556.
49. **Shirakawa, F., and S. B. Mizel.** 1989. In vitro activation and nuclear translocation of NF-κB catalyzed by cyclic AMP-dependent protein kinase and protein kinase C. Mol. Cell. Biol. **9:**2424–2430.
50. **Stone, B. J., C. M. Garcia, J. L. Badger, T. Hassett, R. I. Smith, and V. L. Miller.** 1992. Identification of novel loci affecting entry of *Salmonella enteritidis* into eukaryotic cells. J. Bacteriol. **174:**3945–3952.
51. **Suzuki, T.** 1991. Signal transduction mechanisms through Fc gamma receptors on the mouse macrophage surface. FASEB J. **5:**187–193.
52. **Vaishnav, Y. N., and F. Wong-Staal.** 1991. The biochemistry of AIDS. Annu. Rev. Biochem. **60:**577–630.
53. **Valentin, A., A. von Gegerfelt, S. Matsuda, K. Nilsson, and B. Asjo.** 1991. *In vitro* maturation of mononuclear phagocytes and susceptibility to HIV-1 infection. J. Acquired Immune Defic. Syndr. **4:**751–759.
54. **Yamamoto, K. K., G. A. Gonzalez, W. H. Biggs, 3d, and M. R. Montminy.** 1988. Phosphorylation-induced binding and transcriptional efficacy of nuclear factor CREB. Nature (London) **334:**494–498.