Evidence that the Regulation of Diphtheria Toxin Production Is Directed at the Level of Transcription

J. R. MURPHY,* J. L. MICHEL, AND M. TENG

Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, Massachusetts 02115

Received for publication 16 March 1978

It has been known for several decades that iron inhibits the production of diphtheria toxin by Corynebacterium diphtheriae by preventing expression at maximal levels. We examined the inhibition kinetics of toxin production after the addition of either iron or rifampin to iron-limited cultures of C7(β<sup>ox</sup>−). Iron-mediated inhibition of toxin production was found to be linear within the range of 16 nM to 16 μM. The inhibition kinetics following the addition of iron or rifampin was almost identical. [3H]RNA extracted from iron-limited toxigenic C. diphtheriae was found to hybridize to a greater extent to corynephage β DNA than either [3H]RNA extracted from toxigenic C. diphtheriae before the onset of toxin production or [3H]RNA extracted from nonlysogenic, nontoxigenic C. diphtheriae.

Uchida et al. (28) by the isolation of phage mutants that code for the production of nontoxic proteins that are serologically related to diphtheria toxin have demonstrated that the structural gene for toxin, tox, was carried on the corynebacteriophage β genome. The diphtheria tox gene is expressed independently from all other known β-phage genes and may be expressed from the prophage genome (12), from non-integrated repressed genomes (4), and from replicating genomes during vegetative phage growth (12). Diphtheria tox gene products are only produced at maximal levels when iron is the growth rate-limiting substrate (21). The addition of iron to starved cultures results in a rapid inhibition of toxin production (29). It is clear that different strains of Corynebacterium diphtheriae may vary with respect to their sensitivity to iron inhibition of toxin production, but these differences can almost always be accounted for by the ability of a given strain to grow in the absence of iron (21). This observation has given rise to the suggestion that a corynebacterial factor(s) may be involved in the regulation of the corynebacterial β tox gene. Although it has been known for decades that iron markedly inhibits the production of diphtheria toxin by C. diphtheriae, little is known of the molecular mechanism(s) of iron action.

We have approached the problem of iron inhibition of toxin production from two different directions. The first is the in vitro synthesis of diphtheria tox gene products (16), and the second is the isolation of partial tox constitutive mutant β-phage lysogens of C. diphtheriae (17). The use of Escherichia coli extracts for the in vitro synthesis of diphtheria tox gene products has allowed us to separate bacterial factors that may be involved in iron inhibition of toxin production from β-phage determinants. The addition of 50 to 100 times the concentration of iron, which would almost completely inhibit the synthesis of diphtheria toxin by C. diphtheriae in vivo, had no inhibitory effect on the in vitro synthesis of diphtheria tox gene products in E. coli S-30 extracts. On the other hand, the addition of corynebacterial extracts to the in vitro system results in the specific inhibition of tox expression (16). The results of these experiments suggested a diphtheria tox gene "repressor" activity in extracts of the nonlysogenic, nontoxigenic C. diphtheriae C7(−)ox−. The isolation of mutants, both bacterial (8) and β-phage (17), which produce diphtheria toxin irrespective of the concentration of iron in the growth medium, supports the hypothesis that iron acts as a corepressor in the corynebacterial mediated regulation of the β-phage tox gene.

To further study the inhibitory effect of iron on diphtheria toxin production, we compared the effects of rifampin, an inhibitor of RNA polymerase reinitiation, and iron on the inhibition of diphtheria toxin production. In addition, we report the results of RNA-DNA hybridization experiments in which total [3H]RNA extracted from C. diphtheriae was hybridized to β-phage DNA.

MATERIALS AND METHODS

Bacterial strains. The nonlysogenic, nontoxigenic
C. diphtheriae C7(−)fox− and the lysogenic, toxigenic C. diphtheriae C7(βfox+), and PW8 strains have been previously described (2, 23). Cultures were maintained by lyophilization and passage on chocolate agar plates.

**Bacterial culture medium.** Corynebacterial strains were grown in PT medium of the following composition: 10 g of Casamino Acids (Difco), 10 ml of 10% L-tryptophan, 2.0 ml of solution II (15), 1.0 ml of solution III (15), and 0.5 ml of 0.18% calcium pantothenate. The solution was made up to 1 liter, and the pH was adjusted to 7.2. Aliquots (100 ml) were autoclaved at 100°C for 15 min. To each aliquot 3.0 ml of a sterile 50% maltose-0.5% calcium chloride solution was added aseptically.

C-Y medium for the production of diphtheria tox gene products has been described previously (20). Additional iron was removed from C-Y medium by treatment with Chelex 100 resin (Bio-Rad Laboratories, Rockville Centre, N.Y.). A 5-g portion of Chelex 100 was added to 100 ml of medium and incubated at room temperature for 18 h. Before it was used, the Chelex 100 was removed by centrifugation.

**Diphtheria toxin production.** An overnight culture of C. diphtheriae was diluted with fresh C-Y medium to an optical density at 590 nm (OD_{590}) of 1.5 to 2 and incubated at 34°C with shaking at 240 rpm until an OD_{590} of 4.5 was reached. Bacteria were harvested by centrifugation at 10,000 × g for 10 min at 4°C and suspended in Chelex 100-treated C-Y medium to an OD_{590} of 6 to 9. A 5-ml sample was transferred to a 250-ml acid-washed Erlenmeyer flask and shaken at 240 rpm at 35°C. Samples of 0.4 ml each were periodically removed, and the cells were sedimented in an Eppendorf model 3200 microcentrifuge. Diphtheria toxin released into the culture supernatant fluid was measured by quantitative immunoelectrophoresis as described previously (18). After immunoelectrophoresis in the presence of 0.5 antitoxin units per ml of agarose, rocket precipitation lines were stained by Coomassie brilliant blue after the plate was soaked for 18 h in a 0.04% NaCl-0.04% sodium borate solution and dried. Antigen was quantitated by integration of the rockets formed. Antidiphtheria toxin, lot SA-10, was purchased from the Massachusetts Antitoxin and Vaccine Laboratories, Jamaica Plain, Mass. A plot of the integration units of rockets formed as a function of diphtheria toxin concentration was linear within the range of 5 to 200 ng of toxin. Standards of 100 ng of toxin were run on each immunoelectrophoresis plate.

**Iron determination.** The residual iron concentration in rifampin (P-L Biochemicals, Milwaukee, Wis.) was determined after wet ashing essentially as described by Littlejohn and Raine (9). After complete digestion and neutralization, iron was measured after reaction with 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-3-yl-disulfonic acid (ferrozine) (Aldrich Chemical Co., Milwaukee, Wis.) according to the method of Stookey (27).

**Preparation of [3H]RNA from C. diphtheriae.** The FW8, C7(βfox+) and C7(−)fox− strains of C. diphtheriae were pulse-labeled with 10 μCi of [3H]uridine for 15 min. A 2.5-ml sample of labeled bacterial culture at an OD_{590} of 7 to 9 was rapidly chilled by dilution in ice-cold phosphate-buffered saline containing 20 mM sodium azide. Bacteria were harvested by centrifugation at 10,000 × g for 10 min and the cell pellet was washed twice and resuspended in 10 ml of buffer 1 of Palmiter (19). The bacteria were then disrupted in the French press at 20,000 lb/in². Total RNA was extracted by the phenol/chloroform method of Palmiter (19). Before use all glassware for RNA extraction was acid washed, treated with Siliclad (Clay Adams, Parsippany, N.J.), rinsed with deionized water, and heat treated at 175°C for 6 h to remove residual RNase. Buffers were treated with diethyl oxidoformate (Eastman Chemical Co., Rochester, N.Y.) by the method of Parish (22) to inactivate endogenous RNase.

**Propagation and purification of corynebacteriophage β_{fox}^{+}, β_{fox}^{−}.** The clear plaque-forming mutant of β_{fox}^{+} has been previously described (7). Cultures of C7(−)fox− were grown in 10-liter volumes in a Microferm fermentor (New Brunswick Scientific Co., Edison, N.J.) sparged with air at 4 liters per min, and agitated at 500 rpm at 32°C until the OD_{590} was 0.5. The cultures were then infected with β_{fox}^{+} at a multiplicity of 1 to 2. The absorbance of infected cultures was monitored at 590 nm and, at the onset of mass lysis, sodium citrate was added to a final concentration of 50 mM. Whole cells and debris were removed by centrifugation at 7,000 × g for 20 min and the corynephage were harvested by the method of Yamamoto et al. (31). Corynephage β_{fox}^{−} was purified before the isolation of DNA by centrifugation to equilibrium in a cesium chloride gradient (density, 1.5 g/cm³). The method for DNA extraction has been described previously (16).

**DNA-DNA hybridization.** β_{fox}^{+} DNA used in hybridization studies was treated with 50 μg of self-digested Pronase (Sigma Chemical Co., St. Louis, Mo.) per ml for 2 h at 37°C. The DNA was denatured in a boiling water bath for 10 min and then rapidly chilled in an ice bath. A total of 7.5 μg of DNA was applied to nitrocellulose membrane filters (Millipore Corp., Bedford, Mass.) with gentle filtration. Hybridization was performed at 67°C for 4 to 16 h by the method of Gillespie and Spiegelman (5), as modified by Miller (13).

**RESULTS**

It is widely known that diphtheria toxin is produced at maximal levels when iron becomes the growth rate-limiting substrate for toxigenic C. diphtheriae (21). Figure 1 shows the time course of toxin production by C7(βfox+). Although it has been previously reported that the addition of iron to culture of C7(βfox+) rapidly inhibits toxin production (29), the inhibitory effect of iron has not been titrated. Figure 2A shows the inhibition of toxin production by C7(βfox+) after the addition of iron. Maximal inhibition of toxin production was observed following the addition of iron to 16 μM. Addition of iron to concentrations greater than this did not increase the rate of inhibition. The inhibition of toxin production by C7(βfox+) was linear over a 1,000-fold range in iron concentration (Fig. 2B). Extrapolation of the inhibition curve through 100% suggested that the concentration of iron in
the control flask at the onset of toxin production was approximately $10^{-9}$ M. Even though the addition of 16 μM iron to cultures of C7(β0xx) rapidly suppressed the production of toxin by greater than 90%, there was little if any inhibition of total protein synthesis, as measured by incorporation of $\left[^{14}C\right]$leucine into trichloroacetic acid-precipitable material.

The recent isolation of bacterial mutants (8) which continue to produce diphtheria toxin in the presence of 500 μM iron provides genetic evidence that the β-phage tox gene is affected by a bacterial specific factor. It is not known, however, whether the regulation of tox is mediated directly by an iron complex. Furthermore, it is not known whether the regulation of the diphtheria tox gene is directed at the level of transcription or translation.

To examine these questions we used the antibiotic rifampin as an inhibitor of protein synthesis and followed the kinetics of inhibition of diphtheria toxin production. Since rifampin has been shown to form a stable complex with RNA polymerase (30) and to block reinitiation rather than mRNA chain elongation (6, 26), its action in vivo mimics repression. Moesteller and Yanofsky (14) have shown that the kinetics and order of tryptophan operon repression in E. coli were essentially the same after the addition of either tryptophan or rifampin. If the iron-mediated inhibition of diphtheria toxin production were directed at the level of transcription, one would anticipate that the kinetics of tox repression following the addition of either iron or rifampin should be similar.

The decay kinetics of diphtheria toxin production following the addition of 16 μM iron was almost identical to that observed in rifampin-treated cultures (Fig. 3). The half-life of toxin production after the addition of iron was 6 min compared with a half-life of 8 min after the addition of rifampin. To rule out the possibility that iron contamination of the rifampin was
responsible for the inhibition of toxin production, we measured the concentration of iron in the antibiotic. The final concentration of iron added along with rifampin was calculated to be less than $2 \times 10^{-8}$ M. At this concentration the resulting iron-mediated inhibition of toxin production should be less than 15% of the control.

Next we conducted RNA-DNA hybridization studies using total $[^{3}H]$RNA extracted from the PW8 strain of C. diphtheriae and $\beta$-phage DNA immobilized on nitrocellulose membrane filters. The time course of toxin production by PW8, under the conditions described above, was almost identical to that of the C7($\beta^{ox}$) strain. Toxin production by the PW strain continues for approximately a five- to sixfold increase in bacterial mass after iron becomes depleted (20). $[^{3}H]$RNA labeled and extracted from PW8 before the onset of toxin production hybridized to $\beta$-phage DNA to the same extent as did $[^{3}H]$RNA extracted from the nonlysogenic, nontoxigenic C. diphtheriae C7(-)$^{ox}$ (Fig. 4). In marked contrast, there was an increase in hybridization between $\beta$-phage DNA and the $[^{3}H]$RNA labeled and extracted from PW8 during the period of maximal toxin production. Similar results were obtained in RNA-DNA hybridization experiments using the C7($\beta^{ox}$) strain of C. diphtheriae.

**DISCUSSION**

In this communication we have shown that the inhibitory effect of iron on the production of diphtheria toxin is directly related to the concentration of iron added to limited cultures of C7($\beta^{ox}$) within the range of 16 nM to 16 $\mu$M. The inhibition kinetics of toxin production following the addition of 16 $\mu$M iron suggested a half-life of 6 min for toxin production. In the presence of 1.0 $\mu$g of rifampin per ml, the half-life of toxin production was found to be 8 min. Moesteller and Yanofsky (14) have found that the action of rifampin on the tryptophan operon in E. coli mimics repression by tryptophan with respect to the order and cessation time of $[^{3}H]$uridine incorporation into trp mRNA. While it is not known whether the apparent half-life of toxin synthesis and release into the medium reflects the half-life of diphtheria tox mRNA in vivo, the similarity of inhibition kinetics of diphtheria toxin production following the addition of either iron or rifampin to cultures of C7($\beta^{ox}$) suggests that the primary action of iron may be directed at the level of transcription.

The results obtained in RNA-DNA hybridization experiments further support the hypothesis that iron may act at the level of transcription. Pulse-labeled $[^{3}H]$RNA extracted from toxigenic C. diphtheriae during the period of maximal toxin production hybridized to a significantly greater extent to $\beta$-DNA than either $[^{3}H]$RNA extracted from toxigenic C. diphtheriae before the onset of toxin production, or $[^{3}H]$RNA extracted from nontoxigenic E. diphtheriae.

Other studies of the iron-mediated inhibition of toxin production by C. diphtheriae include those of Matsuda and Barksdale (11, 12). In single-step phage multiplication experiments using the hypervirulent phage $\beta$ht64$^{ox}$, Matsuda and Barksdale found that the addition of 500 $\mu$M iron inhibited toxin production to 10% of the level of toxin produced by a similarly infected iron-starved C. diphtheriae culture. Barksdale and Arden (2) have recently proposed that the inhibitory effect of iron may be at the level of toxin release from C. diphtheriae "involving some sort of feedback control." Were this the case one might expect either the intracellular accumulation of toxin, or the presence of high levels of diphtheria tox mRNA in C. diphtheriae that was not releasing toxin. It is known that diphtheria toxin does not accumulate intracellularly (24), and once corrected for C7(-)$^{ox}$ $[^{3}H]$RNA bound we did not find appreciable
levels of RNA in *C. diphtheriae* prior to toxin production that specifically hybridized to β-

phage DNA.

We have previously proposed a model for corynebacterial regulation of the corynephage β tox gene (17). This model assumes that *C. diphtheriae*, irrespective of its lysogenic state, carries the genetic information for the synthesis of the diphtheria tox aporepressor. In the presence of iron, a repressor-iron complex would form and have specific affinity for the corynephage β tox operator locus. Under conditions of iron limitation, the repressor-iron complex would disassociate and the diphtheria tox gene would become derepressed. The repressor-iron complex would act at the level of transcription as a negative controlling element. Although alternative explanations are possible, the model of corynebacterial regulation of the diphtheria tox gene is consistent with (i) toxin production by lysogenic *C. diphtheriae*, (ii) the iron-insensitive in vitro synthesis of diphtheria tox gene products in S-30 extracts of *E. coli* programmed with β-phage DNA (17), (iii) the constitutive synthesis of diphtheria toxin by the bacterial mutants described by Kanei et al. (8), (iv) the partial constitutive synthesis of toxin by the β-phage mutant lysogen C7(βcr BYU+) and, assuming a limited number of tox repressor molecules per bacterial cell, (v) toxin production in the presence of 500 μM iron by *C. diphtheriae* infected with the hypervirulent phage βhu64 tox+ (11). The function of such a regulatory element in the nonlysogenic, nontoxicogenic C7(−) tox− is unclear. It should be noted that similar models have been proposed for the regulation of enterochelin synthesis in *E. coli* K-12 (3, 25). In this system the synthesis of the iron carrier enterochelin appears to be regulated coordinately with the synthesis of three outer membrane proteins (10).

**ACKNOWLEDGMENTS**

This study was aided by grant PCM76-18924 from the National Science Foundation, and by Public Health Service grant AI112500 from the National Institute of Allergy and Infectious Diseases. J. R. Murphy is the recipient of Public Health Service Career Development Award K04 AI00146 from the National Institute of Allergy and Infectious Diseases.

We thank Patricia Bacha and Jean Nicholas for their critical reading of the manuscript.

**LITERATURE CITED**


