

Insertion or Deletion of the Cheo Box Modifies Radiation Inducibility of *Clostridium* Promoters

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Radiation-inducible promoters are being used in many viral vector systems to obtain spatial and temporal control of gene expression. It was previously proven that radiation-induced gene expression can also be obtained in a bacterial vector system using anaerobic apathogenic clostridia. The effect of radiation inducibility was detected using mouse tumor necrosis factor alpha (mTNF- α) as a model protein under regulation of the radiation-inducible *recA* promoter. In this report, experiments are described in which this *recA* promoter was modified in order to increase radiation responsiveness. Incorporation of an extra Cheo box in the *recA* promoter region resulted in an increase in mTNF- α secretion from 44% for the wild-type promoter to 412% for the promoter with an extra Cheo box after a single irradiation dose of 2 Gy. Deletion of the Cheo box in the promoter region eliminated radiation inducibility. These results prove that the Cheo box in the *recA* promoter is indeed the radiation-responsive element. We also tested whether we could induce the constitutive endo- β -1,4-glucanase promoter (*eglA*) via ionizing irradiation by introducing a Cheo box in the promoter region. While the use of the constitutive promoter did not lead to an increase in mTNF- α secretion after irradiation, the introduction of a Cheo box resulted in a 242% increase in mTNF- α secretion. Reverse transcriptase PCR of RNA samples isolated from irradiated and nonirradiated bacterial cultures demonstrated that the increase in secretion was the result of enhanced transcription of the mTNF- α gene.

In the search for new therapeutic modalities for cancer, gene therapy has attracted enormous interest over the last few years. Many strategies to apply gene therapy have been developed, and even more vectors to deliver the gene of interest have been constructed. However, one of the major pitfalls of gene therapy is still the lack of specificity of gene delivery. Developing a good gene therapy protocol involves the use of a tumor-specific vector system and gene expression limited to the tumor only. This protocol will result in a high therapeutic index: high local tumor control with low systemic side effects.

Recently, the use of bacteria as a tumor-specific protein transfer system has attracted interest. Attenuated *Salmonella* (18, 19), anaerobic *Bifidobacterium* (28), and apathogenic *Clostridium* (2, 5, 6) have been shown to provide selective colonization in tumors. With *Clostridium*, no vegetative bacteria were found in normal tissues (5). Moreover, the use of bacteria as a protein transfer system is very safe, since treatment can be stopped at any time by addition of the appropriate antibiotic (21).

Clostridium can be genetically engineered to express therapeutic proteins such as mouse tumor necrosis factor alpha (mTNF- α) locally in a tumor (23). However, to obtain spatial and temporal control of gene expression, we investigated the use of a radiation-inducible promoter in *Clostridium*. The use of such a promoter would ensure that the therapeutic protein would be expressed only in irradiated tumoral tissues and not

in nontumoral hypoxic tissues, such as abscesses or infarcted tissues. Moreover, protein expression would occur only after radiotherapy, so that gene expression would be switched on and physicians would know from what time on the therapeutic protein would be present (3). With cytotoxic agents such as TNF- α , this protocol would mean a major advantage.

It has been found that the *recA* promoter, belonging to the SOS repair system of bacteria, is induced by radiotherapy at the clinically relevant dose of 2 Gy (13–15). A single dose of 2 Gy significantly increased mTNF- α secretion by recombinant clostridia, by 44%. Moreover, gene activation could be repeated with a second dose of 2 Gy, a result which makes this promoter promising for clinical use, since in patient settings, daily doses of 2 Gy are used (15). However, with this *recA* promoter, there is still basal activity leading to transcription and secretion of mTNF- α under nonirradiation conditions.

In the present report, we investigated whether the Cheo box in the *recA* promoter was responsible for the radiation induction and whether we could increase radiation responsiveness by incorporating an extra Cheo box in the promoter region.

All genes belonging to the SOS repair system are activated by the presence of DNA damage. In nonactivating conditions, a repressor called LexA (for gram-negative bacteria) or DinR (for gram-positive bacteria) binds to a specific operator sequence, called the SOS box or the Cheo box, respectively. When DNA damage occurs, the central protein, RecA, forms a complex with single-stranded DNA that stimulates autoproteolysis of the repressor and thus leads to increased transcription of the SOS genes (1, 10). These genes play a role in repairing the original DNA damage.

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TABLE 1. Shuttle plasmids used in this study^a

Plasmid	Promoter
pIMP- <i>eglA</i> - <i>mTNF</i> - α	<i>eglA</i>
pIMP- <i>eglA</i> <i>Cheo</i> - <i>mTNF</i> - α	<i>eglA</i> with incorporated Cheo box
pIMP- <i>recA</i> - <i>mTNF</i> - α	<i>recA</i>
pIMP- <i>recA</i> <i>extra</i> <i>Cheo</i> - <i>mTNF</i> - α	<i>recA</i> with extra Cheo box incorporated
pIMP- <i>recA</i> <i>deleted</i> <i>Cheo</i> - <i>mTNF</i> - α	<i>recA</i> with Cheo box deleted

^a The *eglA* signal peptide was used in each case for the secretion of mTNF- α .

Our hypothesis was that the addition of a second repressor binding site in the promoter region of a radiation-inducible gene would decrease transcription under basal conditions. After radiotherapy, both binding sites would become free and repression would be absent. In this report, we describe the cloning of the different *recA* promoter-operator mutations and present data on mTNF- α secretion by recombinant *Clostridium acetobutylicum* DSM792 with and without radiation. We also investigated whether we could use the Cheo box to make a constitutive promoter radiation inducible.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. *C. acetobutylicum* DSM792 was grown in 2 \times YT medium (17) at 37°C in an anaerobic system (model 1024; Forma Scientific, Marietta, Ohio) with 90% N₂ and 10% H₂ and with palladium as a catalyst.

For primary vector construction, *Escherichia coli* TG1 (20) was used. This strain was grown in Luria-Bertani broth at 37°C. *E. coli* ER2725 was used for in vivo methylation of plasmid DNA prior to the electroporation of clostridia (8). The *E. coli*-*Clostridium* shuttle plasmid pIMP1 was used as a cloning vector (9).

mTNF- α cDNA was available on plasmid pIG2mTNF (Innogenetics, Ghent, Belgium). Plasmid pHZ117, containing the *eglA* gene of *C. acetobutylicum* P262, was a gift from H. Zappe (29). The *eglA* promoter and signal sequence were used to express and secrete mTNF- α . This chimeric gene construct was present on shuttle plasmid pIMP1, resulting in pIMP-*eglA*-*mTNF*- α (23). The *eglA* promoter in this plasmid was replaced by the *C. acetobutylicum* *recA* promoter, resulting in pIMP-*recA*-*mTNF*- α (15). Table 1 shows an overview of the plasmids used in this study. The *recA* promoter was isolated from chromosomal DNA as previously described (14).

Media were supplemented, when applicable, with erythromycin (25 μ g/ml) or ampicillin (50 μ g/ml).

Mutation of the *recA* and *eglA* promoters, DNA manipulations, and transformation procedures. Introduction and/or deletion of the Cheo box in the *recA* and *eglA* promoters was done using a Quickchange site-directed mutagenesis kit (Stratagene). Table 2 shows the sequences of the wild-type and mutated *recA* and *eglA* promoters at the 3' region. All mutations were introduced in the pIMP1 shuttle vector containing the *eglA* or *recA* promoter followed by the *eglA*-*mTNF*- α fusion gene (Table 1).

For mutation of the *eglA* and *recA* promoters, mutagenic primers containing an extra Cheo box flanked by 10 to 15 bases of the correct sequence were designed. Similarly, mutagenic primers with the desired deletion were developed (the Cheo box is given in bold): primers used to incorporate an extra Cheo box in the *recA* promoter region—5' TATATTGACAAATGAACAAATGTTTCATATAAATTAT ATG 3' and 5' CATATAATTATATGAACATTTGTTTCATTTGCAATATA 3'; primers used to delete the Cheo box in the *recA* promoter region—5' TAA TTATATGTATA_{deletion 12 bp}GAGAGAAAGGTTGG 3' and 5' CCAACCTTT CTCTC_{deletion 12 bp}TATACATATAATTA 3'; and primers used to introduce a Cheo box in the *eglA* promoter region—5' TTTAAGGGACTTTGAACATATG TTCTTGACAAATTAAT 3' and 5' ATTAATTTGTCAAGAACATATGTTCA AAGTCCCTTAAA 3'.

To verify the insertion or deletion of the Cheo box, the DNA fragments containing the introduced mutations were subcloned in pUC19 and the DNA sequence was determined with an automated laser fluorescence ALF Express sequencer (Amersham Pharmacia BioTech). Primers used for sequencing were CY5-labeled M13 forward and reverse primers.

All general DNA manipulations in *E. coli* were carried out as described by

Sambrook et al. (20). Restriction endonucleases and DNA-modifying enzymes were purchased from Roche Diagnostics (Brussels, Belgium), GIBCO BRL (Gaithersburg, Md.), and Eurogentec (Seraing, Belgium) and used as indicated by the suppliers.

Plasmid DNA was isolated from *E. coli* with a Wizard Plus SV miniprep kit (Promega Inc., Madison, Wis.).

E. coli was transformed using chemically competent cells obtained with the RbCl method. Transformation of *C. acetobutylicum* DSM792 was carried out by electroporation as recently described (11).

Irradiation. Recombinant bacteria were grown until early log phase (optical density at 600 nm, ± 0.3). At this time, cultures were divided into two sets, one of which was exposed to radiation while the other was mock irradiated and used as a control. Bacteria were exposed to 2 Gy with a ⁶⁰Co unit at a dose rate of 0.9 Gy/min. This dose of 2 Gy was chosen because it is the dose currently used in most clinical settings. After irradiation at room temperature, bacteria were incubated anaerobically at 37°C, and samples were taken at different time intervals after exposure.

Each experiment was independently repeated three times.

Analysis of mTNF- α secretion. The amount of mTNF- α secreted by recombinant clostridia was quantified using enzyme-linked immunosorbent assay (ELISA) kits (DiaMed EuroGen, Tessenderlo, Belgium). Supernatants taken from irradiated and nonirradiated cultures were diluted 10-fold in phosphate-buffered saline-7.5% bovine serum albumin, and 100- μ l aliquots were placed in wells of a 96-well microtiter plate in duplicate. Further manipulations were done according to the manufacturer's protocol.

Concentrations of secreted mTNF- α were calculated and compared for the irradiated and nonirradiated cultures. The level of radiation-induced mTNF- α production was expressed as the fold increase in mTNF- α concentration of irradiated samples compared with that of the corresponding nonirradiated samples.

Student's *t* test was used for statistical analysis.

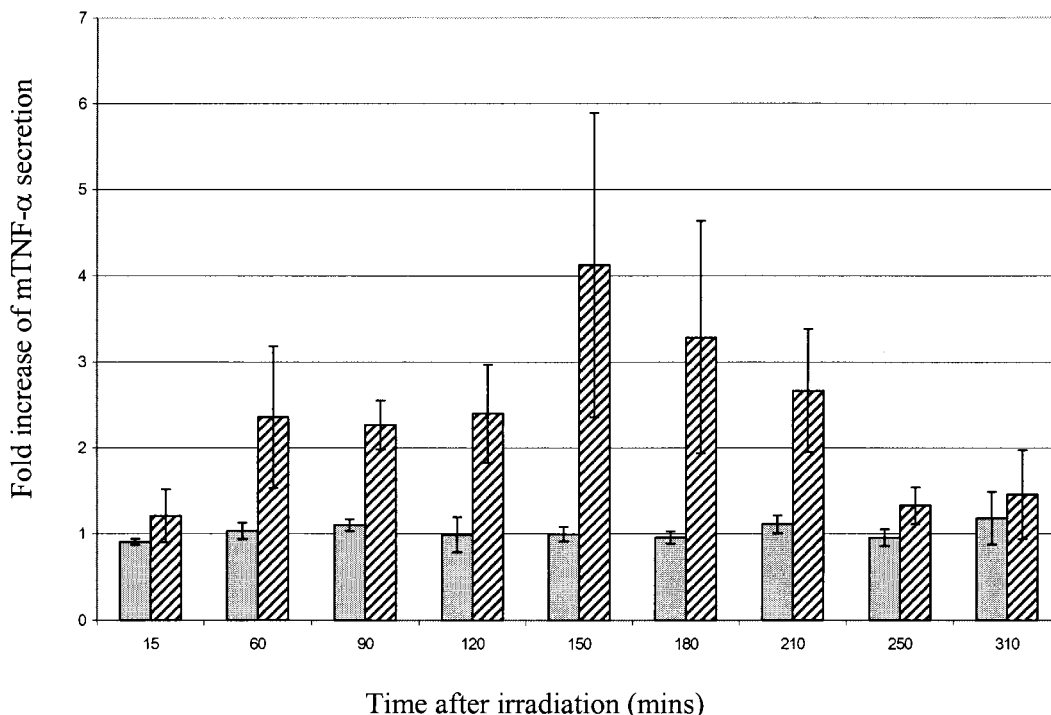
Immunoblot analysis with polyclonal rabbit anti-mTNF- α antibodies was carried out by the method of Van Mellaert et al. (25).

RT-PCR. To prove that the induction of mTNF- α was the result of an increase in promoter activity, reverse transcriptase (RT) PCR (RT-PCR) was performed on RNA isolated from irradiated and nonirradiated bacterial cultures. One hour after radiotherapy, 4-ml aliquots of cultures were taken and RNA was extracted using an RNeasy mini kit from Qiagen (Valencia, Calif.) as previously described (12). The RNA concentration was determined spectrophotometrically. To ensure that there was no DNA contamination which could result in mTNF- α cDNA transcription from the plasmid, 1 μ g of RNA was digested with *Fnu*4HI, which cleaves mTNF- α cDNA at positions 82 and 213, and an additional DNase treatment was carried out. After heat inactivation of the enzymes, 200 U of Moloney murine leukemia virus RT (GIBCO BRL) was added to the RNA together with a mixture containing 0.8 μ l of deoxynucleoside triphosphates (5 mM each), 4 μ l of 5 \times RT buffer, 2 μ l of reverse primer (10 pmol/ μ l), and 2 μ l of dithiothreitol (DTT) (0.1 M) in a total volume of 20 μ l. After 1 h of incubation at 37°C, the resulting cDNAs were amplified using PCR. Five microliters of the RT mixture was added to a mixture containing 8.5 μ l of reverse primer (10 pmol/ μ l), 7 μ l of forward primer (10 pmol/ μ l), 2.5 μ l of deoxynucleoside triphosphates (5 mM each), 0.5 U of JumpStart *Taq* DNA polymerase (Sigma Chemical Co., St. Louis, Mo.), and 4.5 μ l of 10 \times PCR buffer in a total volume of 50 μ l. After 40 PCR cycles (10 min at 95°C, 30 s at 95°C, 2 min at 40°C, 30 s at 72°C, and 5 min at 72°C), 1- μ l aliquots were run on 1% agarose gels. Primers used for the amplification of mTNF- α were as follows: forward primer—5' GTAAGAT CAAGTAGTCAA 3'; and reverse primer—5' CAGAGCAATGACTCCAAA 3'.

To verify the absence of any DNA contamination, all samples underwent the same RT-PCR procedure without the addition of Moloney murine leukemia virus RT. To ensure equal amounts of RNA in all samples, an internal fragment of *C. acetobutylicum* 16S rRNA was amplified using RT-PCR to function as an internal standard. Primers used for the amplification of 16S rRNA were as follows: forward primer—5' GGAGCAAACAGGATTAGATACC 3'; and reverse primer—5' TGCCAACCTCTATGGTGTGACG 3'.

RESULTS

Mutation of the *recA* and *eglA* promoters. After introduction of the wanted mutations in the vectors pIMP-*eglA*-*mTNF*- α and pIMP-*recA*-*mTNF*- α by PCR mutagenesis, mutations were verified by sequence analysis and restriction digestion. There-



promoter	induction factor ± standard deviation									
<i>recA</i> deleted Cheo box	0.91 ±0.04	1.03 ±0.09	1.10 ±0.07	0.99 ±0.21	1.00 ±0.08	0.96 ±0.07	1.11 ±0.10	0.95 ±0.10	1.18 ±0.31	
<i>recA</i> extra Cheo box	1.21 ±0.31	2.36 ±0.83	2.27 ±0.28	2.40 ±0.57	4.12 ±1.77	3.28 ±1.35	2.66 ±0.72	1.33 ±0.22	1.46 ±0.51	

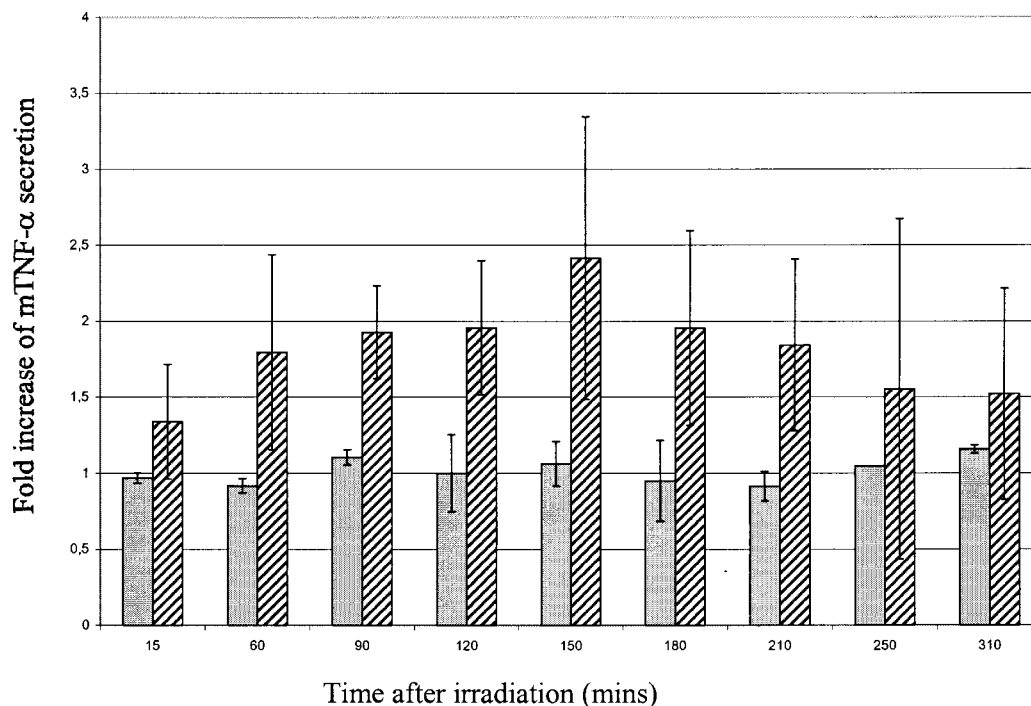
FIG. 1. Fold increase of mTNF-α secretion in *C. acetobutylicum* DSM792(pIMP-*recA*DeletedCheo-mTNF-α) (gray bars) and *C. acetobutylicum* DSM792(pIMP-*recA*extraCheo-mTNF-α) (hatched bars) after a single dose of 2 Gy as a function of time after irradiation. The bars represent data from three independent experiments. Error bars represent standard deviations. Induction factors and standard deviations are shown in the table.

samples showed a weaker band than the irradiated samples, indicating that more mRNA was present in the irradiated samples. For the constitutive *eglA* promoter (Fig. 3, eighth and ninth lanes) and the *recA* promoter with a deletion of the Cheo box (tenth and eleventh lanes), no difference was seen between the irradiated and the nonirradiated samples. For the control samples, both the *recA* promoter with an extra Cheo box and the *eglA* promoter containing a Cheo box showed a weaker band than the corresponding wild-type promoters. This weaker signal can be attributed to lower transcription levels because of higher repression levels under noninducing conditions. The reverse was seen for the *recA* promoter with a deletion of the Cheo box: a stronger signal in the nonirradiated samples for the mutated promoter than for the wild-type promoter. This stronger signal is the result of the absence of repression.

The absence of any band in the samples to which no RT was added confirmed that there was no DNA contamination in any of the samples (data not shown).

DISCUSSION

When DNA damage occurs, bacteria have at their disposal more than 20 genes to repair the original damage. All these genes belong to the so-called SOS repair system (10). These genes have in their promoter region a specific operator sequence, called the Cheo box for gram-positive bacteria and the SOS box or LexA binding site for gram-negative bacteria, to which a repressor binds (1, 7). This repressor is called LexA for gram-negative bacteria and DinR for gram-positive bacteria. When DNA damage is present, RecA will form a complex with single-stranded DNA, and this complex will stimulate autoproteolysis of LexA or DinR, resulting in increased transcription of the SOS repair genes. Both LexA and DinR bind to their operator sequence as dimers (4, 27). The consensus sequence for the Cheo box in gram-positive bacteria is 5' GAAC-N₄-G TTC 3' (1). This consensus sequence is positioned within promoter regions such that the regulatory molecule LexA bound at these sites can interfere with the initiation of transcription



promoter	induction factor \pm standard deviation									
<i>eglA</i>	0.97 ± 0.03	0.92 ± 0.05	1.10 ± 0.05	0.99 ± 0.25	1.06 ± 0.15	0.95 ± 0.27	0.91 ± 0.10	1.05 ± 0.00	1.16 ± 0.03	
<i>eglA</i> with Cheo box	1.34 ± 0.38	1.80 ± 0.64	1.93 ± 0.31	1.96 ± 0.44	2.42 ± 0.93	1.95 ± 0.64	1.84 ± 0.56	1.55 ± 1.12	1.52 ± 0.69	

FIG. 2. Fold increase of mTNF- α secretion in *C. acetobutylicum* DSM792(pIMP-*eglA*-mTNF- α) (gray bars) and *C. acetobutylicum* DSM792 (pIMP-*eglA*Cheo-mTNF- α) (hatched bars) after a single dose of 2 Gy as a function of time after irradiation. The bars represent data from three independent experiments. Error bars represent standard deviations. Induction factors and standard deviations are shown in the table.

by RNA polymerase. Several genes can be found which have two or more putative Cheo boxes, and for those in which repressor binding is proven, the distance between the two boxes is 15 to 16 bp (27).

We investigated whether the Cheo box in the *recA* promoter of *C. acetobutylicum* DSM792 was responsible for induction after ionizing irradiation. We deleted the Cheo box and found that there was no increase in mTNF- α secretion after irradiation, in contrast to the results obtained with the wild-type *recA* promoter when radiation induction was present. When we incorporated a second Cheo box 50 bp upstream of the first, we could increase the radiation responsiveness of the promoter from a 44% increase in the secretion of mTNF- α for the wild-type promoter to 412% for the mutated promoter, in comparison with the results obtained without irradiation. We chose to insert a second Cheo box 50 bp upstream of the first to ensure that there was no spherical hindrance between the two dimers. These results thus demonstrate that the Cheo box in the promoter region of *recA* is indeed the radiation-responsive element and can be used to increase the response after ionizing

irradiation. The addition of a Cheo box also led to a 30% decrease in basal promoter activity.

Moreover, we proved that the introduction of a Cheo box in the constitutive *eglA* promoter caused the mutated promoter to respond to ionizing radiation, in contrast to the results obtained with the wild-type *eglA* promoter. From the literature it is known that the Cheo box is normally located between bp -42 and bp -106 from the start codon (27). Therefore, we introduced a Cheo box 71 bp upstream of the ribosome binding site.

RT-PCR demonstrated that the increase in secretion was the result of increased promoter activity, since higher concentrations of mRNA were present in the irradiated samples. Increased secretion of therapeutic proteins such as mTNF- α in *Clostridium* after irradiation is thus the result of increased activity at the transcriptional level. RT-PCR also demonstrated that under nonirradiation conditions (resembling basal conditions), the addition of a Cheo box resulted in a lower level of transcription and the deletion of a Cheo box resulted in a higher level of transcription after irradiation. These results

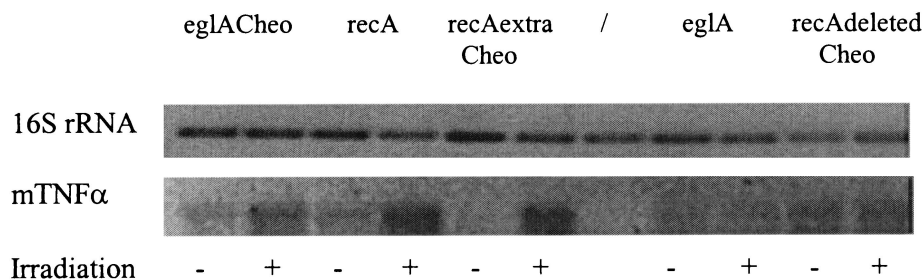


FIG. 3. RT-PCR of irradiated and nonirradiated RNAs extracted from *C. acetobutylicum* DSM792. The upper panel represents the amplification of a 650-bp internal fragment of 16S rRNA which functions as an internal standard to ensure that equal amounts of RNA were used in each RT reaction. The lower panel represents the amplification of a 470-bp internal fragment of mTNF- α . RNA extracted from *C. acetobutylicum* DSM792 was transformed with pIMP-*eglACheo-mTNF- α* (first and second lanes), pIMP-*recA-mTNF- α* (third and fourth lanes), pIMP-*recAextra-Cheo-mTNF- α* (fifth and sixth lanes), pIMP-*eglA-mTNF- α* (eighth and ninth lanes), and pIMP-*recAdeletedCheo-mTNF- α* (tenth and eleventh lanes). The seventh lane shows a positive control for 16S rRNA (PCR performed on chromosomal DNA from *C. acetobutylicum*).

prove that the Cheo box functions as a repressor binding site which becomes free after DNA damage caused by, for example, ionizing irradiation, leading to removal of repression and increased transcription.

Several publications have shown that the Cheo box or LexA binding site is responsible for activation of the SOS repair genes after DNA damage (1). Van der Lelie et al. have demonstrated that the addition of a second LexA binding site in the *E. coli recN* promoter increases inducibility after treatment with genotoxic agents (24).

To our knowledge, this is the first report which proves that the Cheo box is responsible for increased transcription of the *recA* gene after ionizing irradiation in *Clostridium* and can be used to further increase inducibility. Moreover, that fact that we could use radiation to induce the strong *eglA* promoter by introducing a Cheo box in the promoter region implies that the secretion of high doses of therapeutic proteins such as TNF- α can be controlled by ionizing irradiation.

Since the Cheo box is functional in the *eglA* promoter, independent of its natural sequence context, it seems possible to use radiation to induce other clostridial promoters which might be even stronger. We tested only the presence of two Cheo boxes in one promoter, but the addition of more boxes might even increase inducibility and decrease basal activity further.

Systemic administration of therapeutic proteins such as TNF- α is limited due to hepatotoxicity and life-threatening hypotension as major side effects (16). Many groups have been exploring the means to obtain the selective expression of genes locally in a tumor (reviewed in reference 26). Limiting the expression of toxic agents to a tumor cell is extremely important if damage to the surrounding normal tissues is to be avoided. Anaerobic bacteria selectively colonize the hypoxic-necrotic areas of a solid tumor which are absent in healthy normal tissues, and genetically engineered bacteria secrete therapeutic proteins locally in the tumor (22, 23). The use of a radiation-inducible promoter will ensure that no protein is secreted in other necrotic tissues outside the tumor and that secretion will increase after irradiation of the tumor (3). In this manner, the combination of radiotherapy, one of the standard treatment modalities in cancer, and *Clostridium* as a tumor-specific protein transfer system can increase concentrations of therapeutic agents locally in the tumor due to both spatial and temporal control of protein expression. This combination can

result in higher tumor control rates with lower systemic side effects. One advantage of our system is the potential to increase the effectiveness of the *recA* promoter by the addition of Cheo boxes in order to increase responsiveness to ionizing irradiation. When, in the future, more potent clostridial promoters are discovered, the insertion of a Cheo box could make them radiation responsive, increasing the potential use of *Clostridium* as a vector for cancer therapy.

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REFERENCES

1. Cheo, D. L., K. W. Bayles, and R. E. Yasbin. 1991. Cloning and characterization of DNA damage-inducible promoter regions from *Bacillus subtilis*. *J. Bacteriol.* **173**:1696-1703.
2. Fox, M. E., M. J. Lemmon, M. L. Mauchline, T. O. Davis, A. J. Giaccia, N. P. Minton, and J. M. Brown. 1996. Anaerobic bacteria as delivery system for cancer gene therapy: in vitro activation of 5-fluorocytosine by genetically engineered clostridia. *Gene Ther.* **3**:173-178.
3. Hallahan, D. E., H. J. Mauceri, L. P. Sueng, E. J. Dunphy, J. D. Wayne, N. D. Hanna, A. Toledano, S. Hellman, D. W. Kufe, and R. R. Weichselbaum. 1995. Spatial and temporal control of gene therapy using ionizing irradiation. *Nat. Med.* **1**:786-791.
4. Kim, B., and J. W. Little. 1992. Dimerization of a specific DNA-binding protein on the DNA. *Science* **255**:203-205.
5. Lambin, P., J. Theys, W. Landuyt, P. Rijken, A. van der Kogel, E. van der Schueren, R. Hodgkiss, J. Fowler, S. Nuyts, and J. Anné. 1998. Colonisation of *Clostridium* in the body is restricted to hypoxic and necrotic areas of tumours. *Anaerobe* **4**:183-188.
6. Lemmon, M. J., P. van Zijl, M. E. Fox, M. L. Mauchline, A. J. Giaccia, N. P. Minton, and J. M. Brown. 1997. Anaerobic bacteria as a gene delivery system that is controlled by the tumor microenvironment. *Gene Ther.* **4**:791-796.
7. Little, J. W., D. W. Mount, and C. R. Yanisch-Perron. 1981. Purified *lexA* protein is a repressor of the *recA* and *lexA* genes. *Proc. Natl. Acad. Sci. USA* **78**:4199-4203.
8. Mermelstein, L. D., and E. T. Papoutsakis. 1993. In vivo methylation in *Escherichia coli* by the *Bacillus subtilis* phage phi 3T I methyltransferase to protect plasmids from restriction upon transformation of *Clostridium acetobutylicum* ATCC 824. *Appl. Environ. Microbiol.* **59**:1077-1081.
9. Mermelstein, L. D., N. E. Welker, G. E. Bennett, and E. T. Papoutsakis. 1992. Expression of cloned homologous fermentative genes in *Clostridium acetobutylicum* ATCC824. *Bio/Technology* **10**:190-195.
10. Miller, R., and T. Kokjohn. 1990. General microbiology of *recA*: environmental and evolutionary significance. *Annu. Rev. Microbiol.* **44**:365-394.
11. Nakotte, S., M. Schaffer, M. Böhlinger, and P. Dürre. 1998. Electroporation of, plasmid isolation from and plasmid conservation in *Clostridium acetobu-*

- tylicum* DSM792. Appl. Microbiol. Biotechnol. **50**:564–567.
12. Nuyts, S., L. Van Mellaert, P. Lambin, and J. Anné. 2001. Efficient isolation of total RNA from *Clostridium* without DNA contamination. J. Microbiol. Methods **44**:235–238.
 13. Nuyts, S., J. Theys, W. Landuyt, L. Van Mellaert, P. Lambin, and J. Anné. 2001. Increasing specificity of anti-tumor therapy: cytotoxic protein delivery by non-pathogenic clostridia under regulation of radio-induced promoters. Anticancer Res. **21**:857–862.
 14. Nuyts, S., L. Van Mellaert, J. Theys, W. Landuyt, P. Lambin, and J. Anné. 2001. The use of radio-induced bacterial promoters in anaerobic conditions: a means to control gene expression in *Clostridium*-mediated therapy for cancer. Radiat. Res. **155**:716–726.
 15. Nuyts, S., L. Van Mellaert, J. Theys, W. Landuyt, E. Bosmans, J. Anné, and P. Lambin. 2001. Radio-responsive *recA* promoter significantly increases TNF α production in recombinant clostridia after 2 Gy irradiation. Gene Ther. **8**:1197–1201.
 16. Old, L. J. 1985. Tumour necrosis factor. Science **230**:630–636.
 17. Oultram, J. M., Loughlin, T. J. Swinfield, J. K. Brehm, D. E. Thompson, and N. P. Minton. 1988. Introduction of plasmids into whole cells of *Clostridium acetobutylicum* by electroporation. FEMS Microbiol. Lett. **56**:83–88.
 18. Pawelek, J. M., K. Brooks, and D. Bermudes. 1997. Tumor-targeted *Salmonella* as novel anticancer vector. Cancer Res. **54**:4537–4544.
 19. Platt, J., S. Sodi, M. Kelley, S. Rockwell, D. Bermudes, K. B. Low, and J. Pawelek. 2000. Antitumour effects of genetically engineered *Salmonella* in combination with radiation. Eur. J. Cancer **36**:2397–2402.
 20. Sambrook, J. E., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
 21. Theys, J., W. Landuyt, S. Nuyts, L. Van Mellart, E. Bosmans, A. Rijnders, W. Van den Bogaert, A. van Oosterom, J. Anné, and P. Lambin. 2001. Improvement of *Clostridium* tumour targeting vectors evaluated in rat rhabdomyosarcomas. FEMS Immunol. Med. Microbiol. **30**:37–41.
 22. Theys, J., W. Landuyt, S. Nuyts, L. Van Mellaert, P. Lambin, and J. Anné. Specific targeting of cytosine deaminase to solid tumors by engineered *Clostridium acetobutylicum*. Cancer Gene Ther., in press.
 23. Theys, J., S. Nuyts, W. Landuyt, L. Van Mellaert, C. Dillen, M. Böhringer, P. Dürre, P. Lambin, and J. Anné. 1999. Stable *Escherichia coli*-*Clostridium acetobutylicum* shuttle vector for secretion of murine tumor necrosis factor alpha. Appl. Environ. Microbiol. **65**:4295–4300.
 24. Van der Lelie, D., L. Regniers, B. Borremans, A. Provoost, and L. Verschaeve. 1997. The VITOTOX test, an SOS bioluminescence *Salmonella typhimurium* test to measure genotoxicity kinetics. Mutat. Res. **389**:279–290.
 25. Van Mellaert, L., C. Dillen, P. Proost, E. Sablon, R. Deleys, A. Van Broekhoven, H. Heremans, J. Van Damme, H. Eyssen, and J. Anné. 1994. Efficient secretion of biologically active mouse tumor necrosis factor α by *Streptomyces lividans*. Gene **150**:153–158.
 26. Vile, R. G., S. J. Russell, and N. R. Lemoine. 2000. Cancer gene therapy: hard lessons and new courses. Gene Ther. **7**:2–8.
 27. Winterling, K. W., D. Chafin, J. F. Hayes, J. Sun, A. S. Levine, R. E. Yasbin, and R. Woodgate. 1998. The *Bacillus subtilis* DinR binding site: redefinition of the consensus sequence. J. Bacteriol. **180**:2201–2211.
 28. Yazawa, K., M. Fujimori, J. Amano, Y. Kano, and S. Tanuguchi. 2000. *Bifidobacterium longum* as a delivery system for cancer gene therapy: selective localization and growth in hypoxic tumors. Cancer Gene Ther. **7**:269–274.
 29. Zappe, H., W. A. Jones, D. T. Jones, and D. R. Woods. 1988. Structure of an endo- β -1,4-glucanase gene from *Clostridium acetobutylicum* P262 showing homology with endoglucanase genes from *Bacillus* spp. Appl. Environ. Microbiol. **54**:1289–1292.