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Effect of DNA damage on the expression of the chloramphenicol acetyltransferase gene after transfection into diploid human fibroblasts

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ABSTRACT

The activity of the chloramphenicol acetyltransferase (cat) gene after transfection into human fibroblasts has been measured following treatment of the plasmid pRSVcat with either restriction enzymes or ultraviolet light. Restriction enzymes producing single cuts in the plasmid inactivated the expression of the cat gene whether the enzymes cut the plasmid inside the coding region of the gene or several kilobases away from the gene. Ultraviolet light produced a dose-dependent inactivation of the gene. The inactivation curve was steeper if the recipient cell strain was derived from a patient with xeroderma pigmentosum. The findings with this transient expression system contrast with previously reported results of experiments using plasmids which transform cells stably by integrating into the cellular genomic DNA.

INTRODUCTION

The introduction of foreign genes into mammalian cells by DNA-mediated gene transfer has opened up new avenues of research into the mechanisms of gene expression and recombination in mammalian cells, as well as providing a means for cloning mammalian genes. In most studies either the foreign gene is itself a selectable gene inserted into a bacterial plasmid, or it is inserted into or transferred together with a plasmid containing a selectable gene. The foreign DNA is introduced into the cells as a coprecipitate with calcium phosphate, and subsequently selection pressure is applied for the selectable gene. In this type of experiment the foreign DNA usually becomes integrated into the mammalian genome in a small proportion of the cells, and it is then often maintained and expressed for many generations ("stable expression").

Recently Gorman et al. (1,2) have described a different system using plasmids in which the bacterial chloramphenicol acetyltransferase (cat) gene is under the control of viral promoters in pBR322-derived vectors. The structure of one of these plasmids, pRSVcat (2), in which the cat gene

is under the control of the Rous sarcoma virus (RSV) promoter, is shown in Figure 1. When pRSVcat is introduced into mammalian cells the cat gene is expressed transiently within 48h of applying the DNA-calcium phosphate precipitate ("transient expression"). Expression of the gene can be measured using a simple assay for the gene product, chloramphenicol acetyltransferase (CAT) (1). This enzyme is not normally present in mammalian cells. Gorman and coworkers found that many factors which affected the expression of the cat gene in the transient expression system, also affected the efficiency of transfection in stable expression systems in a similar manner (3).

We wished to study the mechanism of DNA repair in a specific gene by transfecting diploid human fibroblasts with the pRSVcat plasmid damaged in different ways. Several reports have shown that linearizing transfecting plasmids outside the gene of interest either increases or does not affect the transfection frequency in stable expression systems (e.g. refs 4,5). Recently Spivak et al. (6) showed that UV-irradiation of the plasmids pSV2gpt or pSV2neo increased the transfection frequency of the gpt or neo genes by a factor of 5 in a stable expression system, irrespective of whether the recipient was a DNA-repair proficient normal human cell line or a repair-deficient cell line derived from a patient with xeroderma pigmentosum. In this paper we report that both a number of restriction enzymes and ultraviolet light cause inactivation of the expression of the pRSVcat plasmid in the CAT transient expression system in primary human fibroblasts.

MATERIALS AND METHODS

The cell strains used in these experiments were the primary human fibroblasts 1BR, GM730, 48BR (normal), and XP4LO (xeroderma pigmentosum - complementation group A) (7). Cells were routinely cultured in Eagle's MEM containing 15% foetal calf serum.

Restriction digestion

The restriction enzymes BglI, BamHI, HindIII, NcoI and PvuII were obtained from either New England Biolabs, P & S Biochemicals Limited or Boehringer Corporation Limited. pRSVcat was digested in 10mM tris-HCl pH7.5, 0.1M-NaCl, 6mM-$MgCl_2$, 1mM-dithiothreitol at 2 units of enzyme per ug DNA for 1h at 37°. Control DNA was incubated in buffer alone. A small sample of digested plasmid was checked on agarose gels to ensure complete digestion, and the rest was ethanol precipitated and dissolved in 2mM-tris HCl pH 7.9, 0.1mM-EDTA.

UV-irradiation

pRSVcat dissolved in 2mM-tris HCl pH 7.9, 0.1mM-EDTA at a concentration of 50ug/ml was UV-irradiated at 254nm at a fluence rate of between 2.5 and 8 Jm^{-2} s^{-1}.

DNA-mediated gene transfer

A modification of the procedure of Graham and van der Eb (8) was used. Cells were seeded at 3 or 5 x 10^5 cells per 9cm plate and incubated respectively for 3 or 2 days. To 40ug plasmid in 0.8ml 2mM tris 0.1mM EDTA was added 0.2ml 1.25M $CaCl_2$. The resulting DNA/$CaCl_2$ solution was added with vigorous bubbling to 1ml of 0.28M-NaCl, 1.5mM sodium phosphate, 25mM Hepes buffer, pH7.1. After 30-45 min duplicate 1 ml samples of precipitate were added to each dish. After incubation at 37° for 6 h, the medium was removed, the cells washed with 5 ml serum-free medium (SFM) and thoroughly drained. 2.5 ml 15% glycerol in 0.14 M-NaCl, 0.75 mM-sodium phosphate, 12.5 mM-Hepes pH7.1 were added for 2 minutes. At this point the glycerol was diluted with 5 ml SFM and the diluted glycerol removed. After a further wash with 5 ml SFM 10 ml fresh medium containing 10 mM-sodium butyrate (9) were added and the cells incubated for 16 h. The following day the medium was replaced with fresh medium without butyrate and incubated for 24 h. Finally the cells were harvested into 1 ml buffered saline by scraping with silicone rubber. They were then centrifuged and the cell pellet either stored at -20° or processed for assay of CAT activity as described below.

CAT assay

The assay was carried out as described by Gorman *et al* (1) with minor modifications. The cell pellet was resuspended in 0.1 ml 0.25M tris-HCl, pH7.8 and subjected to three freeze-thaw cycles (30 sec. in liquid nitrogen, 90 sec. at 37°), and centrifuged in a microfuge at 4° for 5 min. The supernatant contained the CAT activity. The assay for CAT contained in a total volume of 159 ul, 70 ul 0.25M tris-HCl, pH7.8, 50 ul H_2O, 1 ul [^{14}C]chloramphenicol (0.1 uCi; approx. 50 mCi/mmole), 30 ul cell extract and 8 ul 10 mM-acetyl CoA. The reactants were incubated at 37° for 90 min. CAT acetylates chloramphenicol to produce three acetylated derivatives, 3-acetyl chloramphenicol, 1-acetyl chloramphenicol, and 1,3-diacetyl chloramphenicol. After the incubation period, the reaction was stopped by vortexing with 0.7 ml ethyl acetate. Chloramphenicol and its derivatives were extracted into the ethyl acetate layer which was subsequently evaporated to dryness under vacuum. The residue was

dissolved in 30 ul ethyl acetate and spotted onto cellulose TLC plates, which were developed in 95% chloroform: 5% methanol. A mixture containing chloramphenicol and its three acetylated derivatives for use as markers was prepared by reaction of chloramphenicol with acetic anhydride in pyridine, followed by several extractions with ethyl acetate (W. Shaw, personal communication). Material migrating with the marker spots was cut out and the radioactivity counted in toluene/PPO scintillant. Activity is calculated as per cent of [^{14}C]chloramphenicol converted into acetylated derivatives.

RESULTS

1. Parameters of the system

The conditions for optimum activity of CAT in transfected human fibroblasts were similar to those described by Gorman and co-workers (1-3,9). Activity was greatest 48-72 h after transfection, and it increased with increasing amounts of DNA in the calcium phosphate precipitate (unpublished observations). As described by Gorman et al (9), we found that overnight treatment with butyrate following transfection dramatically increased CAT activity (by a factor of approximately 10 - results not shown). Nevertheless the maximum activity found with human fibroblasts was considerably lower than that in most of the cell types investigated by Gorman et al (3). The reaction was linear for 90 min and then ceased, followed by some degradation of the product (not shown). Thus the conditions described in Materials and Methods were adopted as providing maximum CAT activity. Typically about 50% of the chloramphenicol was acetylated if the transfecting plasmid was undamaged, although there was considerable variability, as described below.

A serious problem we encountered in these experiments was the variation between apparently identical samples. Even the same precipitate applied to replicate dishes of cells gave CAT activities which could differ by a factor of as much as 2. Between experiments the variability was even greater. Despite extensive investigations we were unable to pinpoint the source of this variability, except that it occurred at the level of transfection/expression rather than in the CAT assays, ie. duplicate assays with the same cell extract showed very similar CAT activity. Because of this variation all experiments were repeated several times.

2. Restriction of pRSVcat

The plasmid was digested with a number of restriction enzymes, which

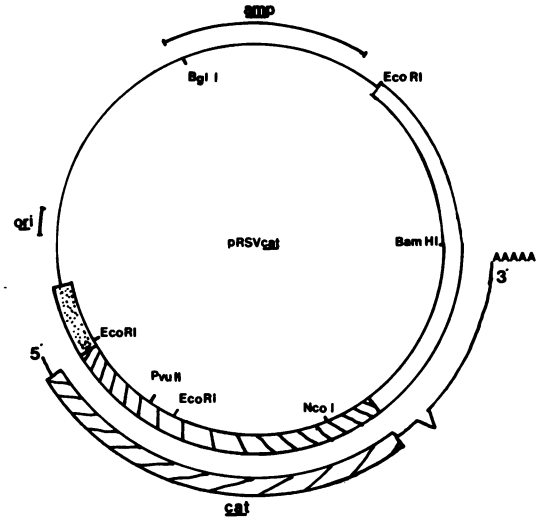

Figure 1. **pRSVcat.** Sequences derived from pBR322 (——), RSV (▪▪▪▪▪), the cat gene (▨), SV40 (▭). The mammalian RNA transcript is also shown (compiled from references 1,2).

made single cuts either within the cat gene (NcoI, PvuII), beyond the polyA addition site (BamHI), or in the plasmid sequences, at least 1.6 Kb away from sequences involved in transcribing the cat gene (BglI) (see Figure 1). Irrespective of the site of action of the enzyme, the treatment always caused greater than 85% inactivation of CAT activity (Figure 2).

3. **UV-irradiation**

pRSVCat was exposed to 0, 100, 300 or 800 Jm^{-2}. Triplicate samples of unirradiated plasmid and duplicates of irradiated plasmid were transfected into various cell strains, and the CAT activity determined as described in Materials and Methods. The activity calculated as a fraction of that of unirradiated plasmid is plotted as a function of UV fluence in Figure 3. UV inactivated the CAT activity in both 1BR and XP4LO cells, the level of inactivation at a given fluence being greater in the XP than in the normal cells. Figure 3 shows inactivation curves for a representative experiment. The ratio of the fluences required to produce 50% inactivation of the cat gene in normal and XP cells was 2.8±0.7 (Mean±SE of 5 experiments).

In order to calculate the number of pyrimidine dimers produced in the plasmid DNA, pRSVcat was exposed to various doses of UV-irradiation,

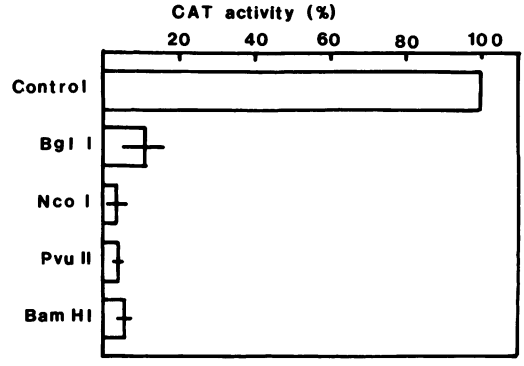

Figure 2. Effect of restriction digestion of pRSVcat on CAT activity. CAT activity is expressed as a percentage of that from undigested DNA in the same experiments. Error bars show SEMs of 3-4 experiments.

followed by treatment with T4 UV-endonuclease (generously provided by Dr.A.A. van Zeeland, Leiden, Netherlands). This enzyme introduces a single-strand nick into the DNA adjacent to each pyrimidine dimer. Estimation of the amount of UV-irradiated closed circular plasmid converted

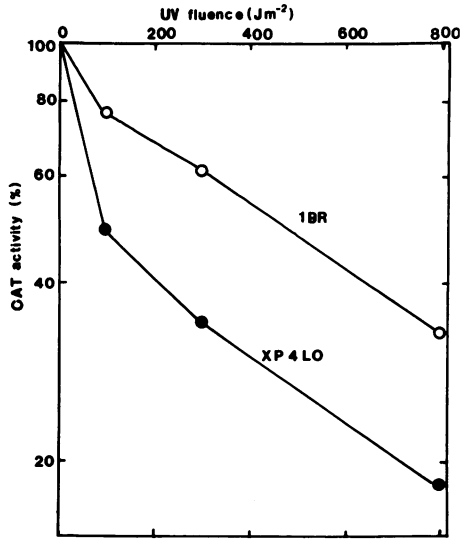

Figure 3. Effect of UV-irradiation of pRSVcat on CAT activity. The activity using irradiated plasmids is expressed as a percentage of that using unirradiated plasmid.

to nicked circles by the UV-endonuclease (10) showed that one pyrimidine dimer was produced per plasmid molecule by a UV fluence of about 12 Jm^{-2}. The transcribed sequence for the cat gene comprises 1.6Kb out of the total 5.0 Kb pairs of the plasmid. Thus the transcribed strand for the gene represents 16% of the total plasmid sequences. Thus one pyrimidine dimer is produced per transcribed strand of the gene at a fluence of about 75 Jm^{-2}.

DISCUSSION

Our results demonstrate that (1) linearizing pRSVcat either inside or outside the coding region of the cat gene, or (2) UV irradiation inactivate the gene as assayed in this transient expression system. These results contrast with the findings with stable expression systems, in which digestion of plasmids with restriction enzymes which cut outside the selectable gene either have no effect or increase the frequency of stable transfectants (eg. see refs. 4-6). In addition, UV fluences producing several pyrimidine dimers in the gpt gene of the plasmid pSV2gpt caused an increase in the frequency of production of stable gpt+ cells, if the plasmid was transfected into either normal or XP cell lines (6). We think it unlikely that these differences result from differences in the plasmids used in the two systems - they are of similar sizes and contain similar sequences - nor do we feel that they can be attributed to the use of different cell types; diploid human fibroblasts in our experiments and various established cell lines in the stable expression systems. J. Burke and A. Mogg have found that Bam Hl digestion of pRSVcat also inactivates CAT expression using COS-7 cells as recipients (personal communication). Neither of the possibilities mentioned above has, however, been excluded.

We consider that the most likely explanation for the differences in the responses of the two types of system lies in their different requirements. The transient expression system presumably depends on direct transcription of the plasmid DNA in the mammalian nucleus. We suggest that transcription relies on the supercoiled structure of the plasmid, which is destroyed on linearization. This interpretation is consistent with results obtained by microinjection of plasmids into Xenopus oocytes. Transcription of the genes encoded by these plasmids was dependent on the plasmids attaining supercoiled closed circular structures in the oocyte nuclei (11, 12). Transcription of an injected linear plasmid was more than 500 times lower than that of a circular plasmid (11). In contrast the

stable expression systems rely on integration of the plasmid DNA into the mammalian genome and subsequent expression of the plasmid-encoded gene. The limiting step in this process is thought to be the integration step, and it is likely that linearization may increase the integration frequency.

Pyrimidine dimers produced in the DNA by UV-irradiation are likely to reduce transcription by acting as transcriptional blocks. In contrast DNA damage may increase the integration frequency. In support of this suggestion Debenham and Webb (13) have found that either X- or UV-irradiation of the recipient cells also increases the transfection frequency in a stable expression system. The important conclusion is that the two systems behave quite differently, and that caution must therefore be exercised in using the CAT system to optimize parameters for a stable expression system (3).

The effect of UV-irradiation on the expression of CAT activity was examined in normal cells and in XP cells from complementation group A, which are very UV-sensitive and almost totally deficient in the ability to excise UV-induced pyrimidine dimers from their DNA (7). The finding of greater inactivation in the UV-sensitive than in the normal cells suggests that the normal cells are able to repair UV damage produced in the transfected plasmid. This is supported by the observation that a fluence of 500-600 Jm^{-2} producing about six pyrimidine dimers in the transcribed strand for the gene, and forty-five dimers in the plasmid, is required to produce one lethal hit in the plasmid, when transfected into normal cells. Evidence for repair of a UV-damaged transfecting plasmid in human fibroblasts has also been obtained by Mooibroek *et al* (14). The transforming activity of plasmid pc194 in *B.subtilis* was inactivated by UV light. Its activity (as assayed in *B.subtilis*) could be restored by transfection into human fibroblasts and recovery of the plasmid from the cells two hours later. It should however be noted that the difference in the inactivation curves of the UV-irradiated plasmid using the normal and XP cells as recipient in our experiments (a factor of approximately 2.8) is much less than the 10-15 fold difference in the inactivation curves of the cells themselves after treatment with UV light (7). This is perhaps not too surprising since production of CAT enzyme molecules requires only transcription and translation of the *cat* gene, whereas cell survival following UV-irradiation of the cells is the end result of a complex series of repair and recovery processes.

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