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IS TERMINAL DEOXYNUCLEOTIDYL TRANSFERASE AN INTRACELLULAR MUTAGEN?*

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We have established stably transformed mammalian cell lines expressing recombinant human terminal deoxynucleotidyl transferase. A 58 kDa, enzymatically active protein is produced by these cell lines. Using the lacI gene of pJYMib shuttle vector as mutagenic target, we found no increase in mutation rates in cells expressing terminal deoxynucleotidyl transferase compared to controls. Our results suggest that the presence of terminal deoxynucleotidyl transferase alone in mammalian cells does not increase mutation rates.

Terminal deoxynucleotidyl transferase (EC 2.7.7.31) catalyzes the polymerization of deoxynucleoside triphosphates onto the 3'-OH end of DNA without template instruction (1). Its unique presence in pre-B and pre-T lymphocytes, as well as the coincidence of ontogeny of terminal deoxynucleotidyl transferase positive cells and development of immunocompetence in animals, suggest that this enzyme might play a role in the generation of immunological diversity (2,3).

In 1974, Baltimore formulated a model for the generation of somatic mutations in the variable (V) region of immunoglobulin genes by terminal deoxynucleotidyl transferase (4). The V regions of immunoglobulin chains were later found to be mostly encoded in germline DNA (5-7), so the role of terminal deoxynucleotidyl transferase is now postulated to be generation of somatic diversification at the V-D and D-J junctions in the heavy chain (where D stands for diversity and J stands for joining) during antibody gene rearrangement (8-10). The mutagenic potential of this enzyme has been demonstrated in in vitro DNA synthesis systems by Kunkel et al. who found that ter-

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minal deoxynucleotidyl transferase is highly mutagenic during the replication of purified phage DNA by eukaryotic DNA polymerase-β (11). The type of mutations found in this in vitro DNA synthesis system resemble those seen during linkage of variable, joining, and diversity segments in the formation of immunoglobulin genes.

The involvement of terminal deoxynucleotidyl transferase in some form of mutagenesis is suggested from both its catalytic properties and its restricted tissue distribution, but demonstration of its mutagenic potential *in vivo* is lacking. In order to examine the mutagenic potential of terminal deoxynucleotidyl transferase in mammalian cells, we have generated several cell lines stably transformed with plasmids that direct the expression of native terminal deoxynucleotidyl transferase in a genetic background that is normally terminal deoxynucleotidyl transferase negative. To measure mutation frequencies in these cell lines, we carried out transient transfection experiments with a *lacI* shuttle system (pJYMib) developed by Lebkowski *et al.* (12) in which the *lacI* gene of *E. coli* is the mutagenic target. If terminal deoxynucleotidyl transferase acts as an intracellular mutagen, it should be possible to observe an increase in I^+ to I^- conversion when the pJYMib DNA is allowed to replicate in cells containing terminal deoxynucleotidyl transferase.

EXPERIMENTAL PROCEDURES

Two recombinant plasmids were constructed with human terminal deoxynucleotidyl transferase cDNA sequences were derived from pT106 and pT223 (13,14). Hind III linkers were added to the full length human terminal deoxynucleotidyl transferase cDNA (2068 bp) and inserted into the Hind III restriction site of shuttle vectors pdBPV-1 to produce pN13 in positive orientation and pN15 in negative orientation with respect to the transcription of the bovine papilloma virus sequences (15-16). The full terminal deoxynucleotidyl transferase cDNA was also inserted into the Hind III cloning site of shuttle vector pSV2-neo located next to the SV40 early promoter in a positive orientation to generated pN17 (pSV2-neo/TdT)(17).

Transformation of mouse mammary tumor cells (C-127-1) with pN13 and pN15 was performed using the calcium precipitation, glycerol shock procedure essentially as described by Graham and Van der Eb (18). Foci in the transfected cell monolayer were scored 3 weeks after transfection. Cells in the foci were removed by localized trypsin treatment and amplified in culture to produce enriched cultures of transformed cells. Pure transformed cell lines were obtained from these enriched cultures by limiting dilution methods.

Transformation of African green monkey kidney cells (COS-7) with pSV2-neo/TdT was carried out by a calcium phosphate procedure described by Chen and Okayama(19) and transformed cells were selected with medium containing

400 µg/ml of Geneticin. Colonies were picked after 3 weeks in the selection medium and cloned by limiting dilution methods. We were not successful in plating out less than 1 cell per well in the cloning procedure, and therefore resorted to repeated plating at 10 cells per well. After 3 months of selection in Gemeticin, several lines were obtained with more than 50% of viable cells producing terminal deoxynucleotidyl transferase. Control lines stably transformed with the parental plasmids (pdBVP-1 and pSV2-neo) were also produced as described above.

Mutation frequency measurements were made on the lacI shuttle vector pJYMib (provided by Dr. M. Calos from Stanford University) (12) in cells containing intracellular terminal deoxynucleotidyl transferase as the potential mutagen. COS-7 cells, COS-7 cells stably transformed with pSV2-neo and COS-7 cells stably transformed with pSV2-neo/TdT were plated at 60% confluency in 100 mm dishes the day before transfection. Each dish was treated with 1 µg of pJYMib using the DEAE-dextran: glycerol shock procedure as described by Graham and Van der Eb (18). After 48 hrs of incubation the pJYMib DNA was isolated from the transfected cells using the Hirt procedure (20). The extracted DNA was treated with restriction enzyme DPN 1 to remove plasmid DNA molecules that had not replicated in the transfected cells. The DNA was transformed into a recA derivative of MC1061F'150kan (i^- and z^-) (provided by Dr. M. Calos from Stanford University), and I^+ and I^- colonies were scored as blue colonies on plates containing XGal (5-bromo-4-chloro-3-indolyl-3-D-galactoside) (21,22).

During formal cloning of transformed cell lines, cytospins of cell cultures were routinely processed for immunocytochemical staining with mouse monoclonal antibodies against human terminal deoxynucleotidyl transferase (23). Enzyme activity was measured in the crude extracts as previously described (2). One unit of terminal deoxynucleotidyl transferase is defined as a nmole of dGMP polymerized in 1 hr.

RESULTS

Expression of human terminal deoxynucleotidyl transferase in non-lymphoid mammalian cells was accomplished with both recombinant plasmids constructed, pdBVP-1/TdT and pSV2-neo/TdT. The growth rates of cells transformed with recombinant vectors are not significant different from those of cells transformed with their corresponding parental plasmids, or untransformed cells.

Three pure transformed lines were isolated with pN15, which contains the terminal deoxynucleotidyl transferase cDNA sequence in opposite orientation with respect to the transcription of the bovine papilloma virus sequence. Immunocytochemical staining of cells in one of these lines with mouse monoclonal antibodies to human terminal deoxynucleotidyl transferase is shown on Fig. 1. Greater than 95% of the cells in the cultures are terminal deoxynucleotidyl transferase positive showing the presence of the enzyme in the

Figure 1. Immunochemical Staining of pdBVP-1/TdT (pN15) Tranformed Cells. Cloned C-127-1 cells stably transformed by pN15 were plated on a glass slide, fixed with paraformaldehyde, reacted sequentially with a mixture of 3 mouse monoclonal antibodies to human terminal deoxynucleotidyl transferase, goat anti-mouse immunoglobulin-gamma, mouse monoclonal anti-horseradish peroxidase:horseradish peroxidase complex, and peroxidase substrate (3-amino-9-ethyl-carbazole:H_2O_2) as described by Di Primio and Bollum (23). Counter stain used was Mayer's hematoxylin.

cytoplasm, nucleus, or both nucleus and cytoplasm. We were not able to isolate any stably transformed cell lines with pN13. Cloning of pSV2-neo/TdT transformed COS-7 cells was more difficult since cloning efficiency was so low. Nevertheless, after 3 months of selection in Geneticin, we were able to obtain several pN17 transformed COS-7 cell lines in which greater than 50% of cells were producing terminal deoxynucleotidyl transferase.

Enzymatically active human terminal deoxynucleotidyl transferase was expressed in both C-127-1 and COS-7 cells stably transformed with recombinant vectors. The levels of terminal deoxynucleotidyl transferase production differ considerably in these cell types. pdBVP-1/TdT transformed lines produce about 300 units per 10^8 cells (a level comparable to that of terminal deoxynucleotidyl transferase positive human lymphoblastoid cell lines), and pSV2-neo/TdT transformed lines produce at levels ranging from 3500 to 9000 units per 10^8 cells. Immunoblot analysis (14) of extracts showed that the terminal deoxynucleotidyl transferase produced in all stably transformed cell lines is 58 kDa, identical to the molecular weight of native human terminal deoxynucleotidyl transferase (data not shown).

Because of the high efficiency of transfection with COS-7 cells using the DEAE-dextran method and high levels of terminal deoxynucleotidyl transferase

Table I. Mutation Rates in lacI Gene in pJYMib in COS-7 Cells Expressing Human Terminal Deoxynucleotidyl Transferase

Test Cell Line	Total Colonies Counted White (I+)	Blue (I-)	I-/I+
COS-7	6.8×10^4	138	2.0×10^{-3}
COS-7/pSV2-neo	1.9×10^5	195	1.0×10^{-3}
COS-7/pN17-40	7.1×10^4	109	1.5×10^{-3}
COS-7/pN17-44	6.2×10^4	19	0.3×10^{-3}

COS-7/pN17-40 and COS-7/pN17-44 are two independent cells lines derived from a mixture of pSV2-neo/TdT (pN17)transfected COS-7 cells. COS-7/pN17-40 and COS-7/pN17-44 cultures showed 56% and 50% terminal deoxynucleotidyl transferase positive cells, repectively. The levels of enzyme expression were 3500 units/10^8 cells and 9000 units/10^8 cells for COS-7/pN17-40 and COS-7/pN17-44 cell lines, respectively.

production in pSV2-neo/TdT transformed cells, experiments to assess the mutagenic potential of terminal deoxynucleotidyl transferase in cells were carried out with two lines of COS-7 cells stably transformed with pSV2-neo/TdT, and two control cell lines: COS-7 cells and COS-7 cells stably transformed with pSV2-neo. The results of one of these experiments are presented in Table I.

The test DNA in this mutagenesis experiment is the lacI shuttle system (pJYMib) containing the lacI gene of E. coli as the mutagenic target and portions of simian virus 40 DNA necessary for replication. In these experiments, the pJYMib is exposed to a potential mutagen in transformed cells for 48 hrs and then returned to E. coli for rapid detection of lacI mutation after isolation of the replicated plasmid DNA. I^- colonies are scored as blue colonies on plates containing XGal. Table I shows a mutation frequency of about 0.2% in lacI when pJYMib is transfected in COS-7 cells and is in the range reported by Lebkowski et al. (12). Compared to the control COS-7 cells, the mutation frequency in lacI of pJYMib is not increased in pSV2-neo transformed COS-7 cells, nor in two independent isolates of pSV2-neo/TdT transformed COS-7 cells. The data in Table I supports the conclusion that the presence of terminal deoxynucleotidyl transferase in mammalian cells does not have a general effect on the mutation rates of the prokaryotic test DNA molecules. Terminal deoxynucleotidyl transferase is therefore not a generally mutagenic enzyme in mammalian cells.

DISCUSSION

The results presented in this communication suggest that the presence of terminal deoxynucleotidyl transferase in mammalian cells does not increase the mutation rate. One technical problem associated with the use of a shuttle vector as mutagenic target is the hugh mutation frequency in DNA transfected

into mammalian cells, two to three orders of magnitude higher than the spontaneous mutation frequency in either mammalian or bacterial cells (12). One might argue that our negative results are due to the high mutation rate caused by the process of transfection of DNA into mammalian cells, and this high mutation rate could mask any increase in mutation rate due to the presence of terminal deoxynucleotidyl transferase in the cell. We cannot rule out this explanation except to point out that the use of the lacI gene in a shuttle vector as the mutagenic target has provided useful information on the specificities of a variety of physical and chemical mutagens in mammalian cells (24-26).

The findings reported here confirm our earlier experiments in bacteria (unpublished). In these experiments we transformed E. coli AB 1157 cells (Arg^-,Pro^-,His^-,Thr^-,Leu^-) with an expression plasmid for human terminal deoxynucleotidyl transferase or a control plasmid and scored reversion rates for the amino acid markers. The reversion rates in E. coli cells expressing terminal deoxynucleotidyl transferase were the same as that of the control cells.

The results presented in this communication along with those obtained in E. coli provide no evidence that terminal deoxynucleotidyl transferase acts as a general mutagen in mammalian or bacterial cells. This does not rule out the possibility that it might have a mutagenic effect on certain specific eukaryotic DNA sequences, or by acting in concert with other lymphoid-cell specific factors.

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