Expression and Processing of Recombinant Human Terminal Transferase in the Baculovirus System*

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Overproduction of human terminal transferase protein has now been accomplished by cloning the coding sequence of human terminal transferase into a baculovirus, where the expression of terminal transferase is under the control of the polyhedrin protein promoter. Two constructs were made, one producing a protein containing the entire terminal transferase fused to 12 amino acids from the NH2 terminus of the polyhedrin protein, and the other producing 58-kDa human terminal transferase. The terminal transferase levels expressed in cells infected with either recombinant baculovirus are around 10,000 units/10⁷ cells at 48 h postinfection, about 200-fold greater than levels expressed in thymus and cultured lymphoblastoid cells.

The chimeric polyhedrin/human terminal transferase protein produced in the infected insect cells has a molecular weight of about 60,000 while the nonfused recombinant human terminal transferase is identical in molecular weight to that present in human lymphoblastoid cells. Both forms of recombinant terminal transferase show immunological and enzymatic activity. When infected cells are pulse-labeled with [³⁵S]methionine at 42–45 h postinfection, about 10% of newly synthesized protein is terminal transferase. Both forms of terminal transferase are phosphorylated in recombinant virus-infected cells as demonstrated by pulse-labeling infected cells with [³²P]-inorganic phosphate and isolation of labeled terminal transferase peptides by immunoprecipitation.

Terminal transferase (TdT) activity was first described in 1960 (1) as a side reaction present in partially purified DNA polymerase preparations from calf thymus glands. The activity catalyzes the addition of deoxynucleotides onto the 3' hydroxyl end of DNA initiators without template direction (2). TdT was originally thought to be related to DNA polymers by immunofluorescent staining (5). We have used immunofluorescent staining routinely to monitor the production of TdT during the infection process. Intense nuclear immunofluorescence is detected in both recombinant virus-infected cells at about 16 h after infection (data not shown). In the fluorescence photomicrographs of AcNPV373/TdT311 infected SF-9 cells presented in Fig. 1, about 27% of the cell population is TdT positive at 24 h postinfection (Fig. 1A), and this increases to more than 95% after 45 h (Fig. 1B). Although Fig. 1 suggests only cytoplasmic fluorescence for

EXPERIMENTAL PROCEDURES

Production of Recombinant Baculoviruses Expressing Enzymatically Active Human TdT—Both of our constructs contained the human TdT cDNA sequence downstream from the polyhedrin protein promoter. One variation of recombinant virus contained DNA sequence coding for 12 NH2-terminal amino acids of the polyhedrin protein (AcNPV360/TdT331) producing a fusion protein and the other only the human TdT sequence (pAcNPV373/TdT327). DNA sequencing of the constructs in the transfer vectors confirmed the predicted sequences. Insect cells infected with the recombinant viruses produce proteins that are easily detected by rabbit antibodies to calf TdT by immunofluorescent staining (5). We have used immunofluorescent staining routinely to monitor the production of TdT during the infection process. Intense nuclear immunofluorescence is detected in both recombinant virus-infected cell systems at about 16 h after infection (data not shown). In the fluorescence photomicrographs of AcNPV373/TdT327-infected SF-9 cells presented in Fig. 1, about 27% of the cell population is TdT positive at 24 h postinfection (Fig. 1A), and this increases to more than 95% after 45 h (Fig. 1B). Although Fig. 1 suggests only cytoplasmic fluorescence for

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1 The abbreviations used are: TdT, terminal transferase; SDS-PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate; TNM-FH, Trichoplusia ni medium-Fred Hinks. The abbreviations also include those of nucleotides and polynucleotides as recommended by International Scientific Unions (1970 J. Biol. Chem. 245, 5171–5176).
FIG. 1. Immunofluorescent staining of AcNPV373-TdT327-infected SF-9 cells. Production of TdT in AcNPV373/TdT327-infected SF-9 cells was monitored by immunofluorescent staining of infected cells with rabbit anti-calf thymus TdT antibody detected with fluorescence isothiocyanate-conjugated (Fab')2 of goat anti-rabbit IgG. Panel A is a photograph of TdT immunofluorescence from AcNPV373/TdT327-infected cells at 24 h after infection. About 27% of cells present in this culture showed intense TdT staining. Panel B shows TdT immunofluorescence of the same culture at 48 h after infection (over 90% TdT-positive).

TABLE I
Production of TdT enzyme activity in recombinant baculovirus-infected cells

| Postinfection time (h) | AcNPV360/TdT331 Enzyme activity | AcNPV373/TdT327 |
|------------------------|----------------------------------|-----------------|
| 0                      | <5 units/mg protein              | <5              |
| 1                      | <5                               | <5              |
| 12                     | 640                              | 670             |
| 24                     | 6100                             | 4400            |
| 36                     | 8010                             | 7720            |
| 48                     | 4660                             | 2680            |

*a unit is defined as 1 nmol of dGMP incorporated into acid-insoluble material/h.

TdT in infected cells, this is largely an artifact of the method of exposure which integrates light from the most intense source of light. It would appear that as infected cells accumulate TdT, the nucleus becomes saturated and the bulk of the TdT is accumulated in the cytoplasm of cells overproducing TdT. Cell death begins to occur after 36 h of infection.

The results of TdT enzyme assays on the infected cells are presented in Table I. Catalytically active TdT was readily detected 24 h after infection with either recombinant virus. These activity levels increased and reached a maximum at about 48 h. Although immunofluorescence staining of infected cells indicated that the AcNPV373/TdT327-infected culture contained 27% TdT positive cells at 24 h after infection while the AcNPV360/TdT331-infected culture contained 15% TdT positive cells, the enzyme activity levels present are comparable. This observation suggests that the chimeric protein is produced more effectively than the recombinant TdT without any leading polyhedrin amino acid sequence. At about 48 h postinfection, 10–20% of the cells in the culture are nonviable, but all remaining viable cells are TdT positive. After 72 h of infection, about 50% of cells in the cultures are dead. High levels of enzyme activity are present in the culture medium at this time.

The level of enzyme activity produced in the recombinant virus-infected cells at 48 h postinfection is about 10,000 units/10⁷ cells. Since the level of TdT activity in human lymphoblastoid cells is 50 units/10⁷ cells, expression of TdT in the recombinant virus-infected insect cells is about 200-fold greater. The specific activity of the enzyme expressed on a per cell basis in the crude extracts of recombinant virus-infected cells is about 50–100-fold greater than that of TdT positive lymphoid cells due to the larger size of the SF-9 cells.

Production of Immunoreactive TdT Peptides in Recombinant Virus-infected Cells—Immunofluorescent staining for TdT was our principle method for screening and plaque purification of the recombinant viruses. The nature of the immunoreactive TdT peptides produced by the infected cells was examined by subjecting recombinant virus-infected cell lysates to analysis by SDS-PAGE and immunoblotting. These results are presented in Fig. 2. A peptide identical in molecular structure...
weight to TdT isolated from human lymphoblastoid cells (KM-3 cells) can be detected in AcNPV373/TdT327-infected cells (lanes C and C'). A peptide slightly larger (about 60 kDa) is found in AcNPV360/TdT331-infected SF-9 cells (lanes B and B'). No immunoreactive material is seen in the wild-type baculovirus-infected cells (lanes A and A'). While AcNPV373/TdT327-infected cultures have a higher percentage of cells producing TdT by immunofluorescence, the amount of TdT peptide produced is clearly higher in cells producing chimeric protein (lanes B and B') than the recombinant TdT (lanes C and C'). The major TdT peptide produced in each case is, however, that expected from the predicted structure.

Protein Synthesized in Recombinant Virus-infected SF-9 Cells—Fig. 2, A–C, depicts total protein present in infected cells as visualized by Coomassie Blue stain. Pulse-labeling of infected culture with [35S]methionine allows an estimation of the relative amounts of different proteins being synthesized in the infected cultures and shows quite a different picture. Autoradiographs of the pulse-labeled proteins are shown in Fig. 3. Cell cultures labeled with [35S]methionine for 1 h at 42 h after infection under the conditions described under "Experimental Procedures" show only small amounts of labeled peptide in any of the four cultures (two experimental, two control). This low level of labeling is probably a result of dilution by the intracellular pool of methionine in the rather large SF-9 cells. Nevertheless, even in these samples, the predominant peptide synthesized in the wild-type virus infected cells is the polyhedrin protein (lane B), and the major peptide synthesized in the recombinant infected cells is the chimeric or normal TdT protein (lanes C and D). When labeling was allowed to continue for 3 h a clearer picture is obtained. A 58-kDa peptide is one of several major peptides synthesized in AcNPV373/TdT327-infected cells (lane D'), but the relative level of production of this peptide is lower than the production of the 60-kDa peptide in AcNPV360/TdT331-infected cells (lane C'), even though TdT immunofluorescence showed that all cells in these two cultures were producing TdT at this time in the infection (42 h).

Phosphorylation of Recombinant TdT in Infected Insect Cells—We have previously shown that the 58-kDa TdT can be phosphorylated using the catalytic subunit of beef heart cAMP-dependent protein kinase while the 56-kDa species was not phosphorylated under the same in vitro conditions (21). Since the 58-kDa species differs from the 56-kDa species by lacking 23 N-terminal amino acids (12), we concluded that the phosphorylation site on TdT is either Ser at position 7 or Thr at position 19 in the human TdT sequence (14, 22). These two potential phosphorylation sites flank a putative nuclear localization sequence located at positions 11–17 (23). Experiments on labeling of human lymphoblastoid cells with [32P]phosphate showed that TdT is normally phosphorylated in lymphoblastoid cells (22).

In order to determine whether the recombinant TdT molecules produced in infected insect cells are phosphorylated, we labeled infected cultures with [32P]phosphate for 1 and 3 h starting at 42 h after infection. TdT was isolated on an immunoaffinity adsorbent and analyzed by SDS-PAGE and autoradiography. The data obtained after 1 h of labeling is presented in Fig. 4. Coomassie Blue staining of the polyacrylamide gel shows only the heavy and light chain of mouse IgG in uninfected SF-9 cells (lane A) and wild-type baculovirus-infected SF-9 cells (lane B). An additional 60-kDa peptide is present in the sample isolated from AcNPV360/TdT331-infected cells (lane C), and a 58-kDa peptide is present in the sample isolated from AcNPV373/TdT327-infected cells (lane D). Autoradiography of the dried gel shows that both forms of TdT produced by recombinant virus-infected cells are phosphorylated.

![Fig. 4. Phosphorylation of TdT in recombinant virus-infected SF-9 cells. Infection of SF-9 cultures, incubation in [32P] phosphate-containing medium, preparation of cell extracts and immunofluorescence of TdT from cell extracts are described under "Experimental Procedures." After separation on SDS-PAGE, the gel was stained with Coomassie Blue and phosphoproteins were detected by autoradiography. Lane A, staining pattern of peptides bound to mAb/TdT-Protein A-Sepharose in the uninfected SF-9 cell extract, and lane A', its autoradiogram. Lane B, staining pattern of peptides bound to Mah/TdT-Protein A-Sepharose in AcNPV-infected cell extract, and lane B', its autoradiogram. Lane C, staining pattern of peptides bound to mAb/TdT-Protein A-Sepharose in AcNPV360/TdT331-infected cell extract, and lane C', its autoradiogram. Lane D, staining pattern of AcNPV373/TdT3327-infected cell extract, and lane D', its autoradiogram. The two Coomassie Blue-stained peptides of about 55 and 25 kDa present in all samples are the heavy and light chains of mouse IgG, presumably released from the mAb/TdT-Protein A-Sepharose.](image)
phosphorylated (lanes C' and D'). These results suggest that the enzyme required for phosphorylation of TdT is present in the insect cells. Thus, phosphorylation of TdT in unrelated host cells provides no new clues about the biological specificity of the phosphorylation and its relationship to the biological function of TdT.

The amounts of radioactivity incorporated into TdT in 1-h samples is virtually the same as that found after 3 h of labeling (data not shown). This result perhaps indicates that the equilibration of extracellular [*32P*]phosphate with intracellular phosphate is rapid and that rapid equilibrium is established in modification of TdT by phosphorylation.

**DISCUSSION**

TdT is unique in its ability to generate DNA sequences in the absence of template direction (2). It is present in immature cells of the hematopoietic system in those cells undergoing immunoglobulin and T-cell receptor gene rearrangements, suggesting that TdT might play a role in somatic generation of diversity (24–27). Although a great deal is now known about the enzymatic activity of TdT with various substrates, synthetic initiators, and DNA fragments, we have no direct knowledge of the activity of TdT on the DNA gene sequences that it is presumed to act on in the cell. This has been due partly to the lack of suitable DNA substrates and partly to lack of appropriate enzyme species (14, 28). The successful cloning and expression of TdT sequence in *E. coli*, followed by the present development of a useful expression system, provides a workable solution to the latter problem. Knowledge of protein sequence deduced from DNA sequence also allowed us to postulate several domains of the enzyme protein (14), and the activities and functions of these domains can now be verified.

Other laboratories studying the diversification occurring during the recombinational events in immunoglobulin gene rearrangement have produced bacterial plasmids containing the minimum DNA sequences required for site-specific recombination (29, 30). The ability to produce various recombinant TdT sequences with defined domains should allow us to systematically study the possibility of interaction of TdT with other components of the recombinant system that brings about immunoglobulin gene rearrangement.

Since our initial success in cloning TdT we made numerous unsuccessful attempts to overexpress it in *E. coli*. Constructs made with TdT coding sequence under the control of P-trp, P-trc, and bacteriophage λ PL produced catalytic activity and immunoreactive peptides TdT in *E. coli* cells, but the levels of enzyme activity produced from these overexpression plasmids never exceeded from that obtained from pT223 (P-lac) containing *E. coli* cells (12). The immunoreactive peptides detected in the strong overproducing systems were heterogeneous in molecular weight, suggesting intracellular degradation of recombinant TdT peptide, or restarts on internal codons of the cDNA. The reason for our failure to overproduce in bacteria is unknown but may be related to mismatches in the translational machinery in *E. coli* and eukaryotic cells.

If the difficulties in overexpression of human TdT cDNA in *E. coli* is caused by mismatch of codon frequencies and tRNA pools, this might be circumvented by using one of the eukaryotic expression systems now available. Several eukaryotic proteins have been successfully overproduced in the baculovirus system by placing coding sequences under the control of promoter and leader sequences of the baculovirus polyhedrin protein (15, 16, 31–34). This work presents evidence that useful production of enzymatically active human TdT can be obtained from recombinant baculovirus-infected insect cells. Our plaque-purified recombinant viruses are stable, and the time course of the infection cycle, as well as titers of recombinant virus stocks, are comparable to those of the wild-type virus. These observations suggest that TdT is not a generally mutagenic DNA polymerase, since that property would result in selective pressure against the recombinant virus.

The levels of recombinant TdT we have produced in infected cells constitute about 1% of the total cell protein. This level of TdT expression will allow us to produce sufficient amounts of full-length protein and protein derivatives for studies that would not be contemplated with the protein levels present in any natural expression system. Since the insect virus system appears to phosphorylate the recombinant TdT, studies on the role of phosphorylation in the regulation of activity and the stability of TdT proteins can now be carried out. We are currently constructing recombinant viruses expressing truncated TdT molecules lacking the first 20 NH₂-terminal amino acids. The putative phosphorylation site(s) and the nuclear localization sequence of TdT are located within this NH₂-terminal sequence. A comparison of the properties of full-length and truncated molecules should provide us with some idea on the function of the NH₂-terminal domain of the TdT protein. Comparison of DNA binding, protein binding, and enzymatic properties of recombinant TdTs with deletions in different domains of the molecule should eventually allow us to systematically identify the enzymatic and biological activities of this important protein.

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Recombinant Human Terminal Transferase

Differential Material 58

Expression and Processing of Recombinant Human Terminal Transferase in the Mammalian System

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Experimental Procedures

Materials: Recombinant adenovirus Ad 56, L and RN 61 were purchased from Bethesda Res. CD1. Restriction endonucleases Eco RI and Xho I and bacteriophage buta DNA ligase were from New England Biolabs. cDNA cDNA was obtained from several sources, 0.15 M phage RF 114 and the large fragment of pBR 322 were purified from female Res. In, 15% polyethylene glycol precipitation of RF 114 phage RF 114 was obtained by electroporation of a female Res. Female Res. Female Res. Female Res. Female Res. Female Res. Female Res. Female Res. Female Res. Female Res. Female Res. Female Res. Female Res. Female Res. Female Res. Female Res. Female Res. Female Res. Female Res. Female Res. Female Res. Female Res. Female Res. Female Res. Female Res. Female Res. Female Res. Female Res. Female Res. Female Res. Female Res. Female Res. Female Res. Female Res. Female Res. Female Res. Female Res. Female Res. Female Res. Female Res. Female Res. Female Res. Female Res. Female Res. Female Res. Female Res. Female Res. 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