Expression of enzymatically active reverse transcriptase in *Escherichia coli*

(gene fusions/RNase H/cDNA synthesis)

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ABSTRACT   Reverse transcriptase of murine retroviruses is a monomeric protein of ≈80,000 daltons, which is encoded by the central portion of the viral *pol* gene. To prepare large quantities of the enzyme, we have constructed gene fusions between the *trpE* gene and portions of the *pol* gene of Moloney murine leukemia virus. The inserted *pol* gene sequences include the entire coding region for the mature enzyme and various amounts of additional coding sequences. Many of these constructs express high levels of reverse transcriptase activity even though the NH_2 and COOH termini of the protein product only approximate the correct termini of the authentic protein.

The synthesis of retroviral DNA is catalyzed by the enzyme reverse transcriptase, encoded by the viral *pol* gene. The enzyme can efficiently utilize either RNA or DNA templates for DNA synthesis by the elongation of a primer bearing a paired 3' hydroxyl terminus (1–6). The *pol* gene is initially expressed in the form of a large polyprotein precursor, containing sequences encoded by the *gag* gene fused to sequences encoded by the *pol* gene; proteolytic processing is required to remove the *gag* sequences and to excise the mature products from the *pol* sequences (7). In the murine retroviruses, the *pol* sequences are processed to three nonoverlapping products: a small protein encoded by the 5' end of the gene, the protease needed for *gag* and *pol* processing (8); reverse transcriptase, the largest protein, encoded by the middle of the gene; and a protein encoded by the 3' end, apparently involved in integration of the provirus (9, 10). To facilitate characterization of these proteins, we have generated constructs that express portions of the *pol* gene of the Moloney murine leukemia virus in *Escherichia coli*. Several of these constructs were found to express active reverse transcriptase.

MATERIALS AND METHODS

Bacteria and Plasmids. *E. coli* strain HB101 (*recA13⁻*, *hsdR⁻*, *hsdM⁻*, *lacY1*, *SupE44*) was used as the host for most experiments (11). Strain C2110 (*his⁻*, *rha⁻*, *polA1⁻*) was the gift of D. Figurski. The *trpE* fusion vector pATH1, was the generous gift of T. J. Koerner and A. Tzagoloff. The *pol* gene was derived from plasmid pT11 (12).

Enzymatic Reactions. DNA fragments were purified by agarose gel electrophoresis and recovered with glass powder (13). DNA was treated with exonuclease BAL 31, nuclease S1, and T4 DNA ligase as described (14). Reverse transcriptase assays measured the incorporation of radioactive dTTP into homopolymer on synthetic templates (15).

DNA Sequencing. The bases in pSHNB6 flanking the site of deletion were determined by the procedure of Maxam and Gilbert (16). Plasmid DNA was 5'-end-labeled at *Hinc*II sites

with polynucleotide kinase and cleaved again with *Rsa* I to prepare a 240-base-pair (bp) fragment labeled at one end.

Preparation of Crude Lysates. These procedures were modifications of the method of Kleid *et al.* (17). Cultures (0.5 ml) were grown to stationary phase in M9 medium (18) with 0.5% Casamino acids/thiamine (10 μg/ml)/tryptophan (20 μg/ml)/ampicillin (50 μg/ml), diluted 1:10 into medium without tryptophan, and grown for 1 hr at 30°C. The cells were induced by addition of indoleacrylic acid to 5 μg/ml, grown an additional 2 hr, and harvested by centrifugation. Preparation of total protein extracts for enzymatic assays, and the subsequent separation of proteins into soluble and insoluble fractions were carried out as described (17). Extracts made by the dilute lysis procedure were prepared from small cultures lysed in 1/10th vol, and extracts made by the concentrated lysis procedure were made from 500-ml cultures lysed in 1/200th vol.

Immunoprecipitations. Cells were labeled by addition of [³⁵S]methionine to 40 μCi/ml (1 Ci = 37 GBq) at the time of induction, treated with lysozyme as described above, and lysed with buffer A (1% Triton X-100/0.5% sodium deoxycholate/0.1% $NaDodSO_4$/10 mM sodium phosphate, pH 7.5/0.1 M NaCl) for 15 min at 0°C. After addition of fixed *Staphylococcus aureus* cells (Pansorbin, CalBiochem), the lysate was clarified (45,000 rpm; 90 min), and incubated with antiserum overnight. The immune complexes were adsorbed to fixed *S. aureus* cells for 1 hr at 0°C, collected, and analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis as described (14).

Partial Purification of pSH1 and pSHNB6 Proteins. HB101 cells bearing pSH1 or pSHNB6 were grown to stationary phase at 37°C and induced as described above. The cells were collected by centrifugation, washed, resuspended in 1/200th vol of buffer (50 mM Tris·HCl, pH 7.5/0.5 mM EDTA/0.3 M NaCl), and treated with lysozyme (1 mg/ml) at 0°C for 30 min. The cells were lysed with Nonidet P-40 (NP40) (0.2%), and the lysate was made 1 M in NaCl, clarified at 8000 × g for 30 min, and dialyzed against buffer B [50 mM Tris·HCl, pH 8.0/1 mM EDTA/1 mM dithiothreitol/10% (vol/vol) glycerol] containing 0.1% NP40 and 25 mM NaCl. DNA was precipitated by the addition of 0.3 vol of streptomycin sulfate solution (5% in buffer B containing 25 mM NaCl), and the supernatant was applied to a DEAE-cellulose column (DE52; Whatman) equilibrated with buffer B containing 25 mM NaCl. The activity was eluted with buffer B containing 0.2 M NaCl.

Ammonium Sulfate Fractionations. Activity eluted from DEAE cellulose columns was precipitated by the addition of solid ammonium sulfate to the appropriate concentration. The solution was stirred for 1 hr, and the precipitate was pelleted (11,000 rpm; 30 min).

Abbreviations: bp, base pair(s); MalNEt, *N*-ethylmaleimide.

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RESULTS

Construction of trpE–pol Gene Fusion. In our initial effort to express reverse transcriptase, we chose a fragment from the central portion of the pol gene of a cloned biologically active copy of the Moloney murine leukemia virus genome (12). Sac I cleaves within the 5' portion of the pol gene encoding the viral protease; a deletion mutation near this site does not affect reverse transcriptase activity (8). HindIII makes a single cleavage in the 3' portion of the pol gene, and deletions at this site also do not affect production of the enzyme (9). The 2.5-kilobase fragment produced by cleavage with Sac I and HindIII was isolated and inserted into the polylinker sequence of the expression vector pATH1 (T. J. Koerner and A. Tzagoloff, personal communication). The resulting plasmid, pSH1, contained the trp promoter, a portion of the trpE gene, and the coding region for the central portion of the pol gene appended in the correct reading frame (Fig. 1). The gene product would contain 36,200 Da of the trpE polypeptide joined to 87,700 Da of pol protein; translational termination of the fusion protein would occur at an amber codon in the vector immediately downstream of the pol sequences.

In an attempt to form smaller protein products that would more closely resemble the authentic enzyme, we modified the initial construct. We removed the bulk of the trpE sequences and portions of the 5' end of the pol gene by creating a series of deletions in the pSH1 plasmid (Fig. 1). pSH1 DNA was cleaved near the 5' end of the trpE gene with Nru I, and treated with the exonuclease BAL 31; the DNA was recleaved with Sac I at the 3' end of the trpE gene, and the termini were blunt ended with nuclease S1. The linear DNA was purified, recyclized with T4 DNA ligase, and used to transform HB101 cells to ampicillin resistance. Analysis of the DNA from several colonies showed

that various amounts of the trpE and pol genes had indeed been removed (data not shown).

The trpE–pol Fusions Induce Reverse Transcriptase Activity. Cells containing pATH1 and pSH1 were starved for tryptophan, harvested, and lysed, and the crude extracts were tested for reverse transcriptase activity. The assay measured the incorporation of radioactive dTTP on poly(rA) primed with oligo(dT) and was similar to assays previously used to detect the viral enzyme (15). Extracts prepared from HB101 cells, or from cells bearing pATH1, showed significant basal activity in the assay. The bulk of this background activity is attributable to DNA polymerase I, which is known to exhibit reverse transcriptase activity (19). Cells bearing plasmid pSH1, however, consistently showed 4- to 6-fold higher activity than the control cells (Fig. 2A; Table 1). The level of activity per ml in these crude extracts was considerably higher than that in viral harvests taken from infected NIH/3T3 cell lines. Recovery of the activity in the soluble fraction required the presence of nonionic detergent and high salt concentrations (data not shown).

To test whether the increased activity could be attributed to an increase in the level of DNA polymerase I, the sensitivity of the activity to the sulfhydryl reagent N-ethylmaleimide (MalNEt) was determined. The authentic murine reverse transcriptase is exquisitely sensitive to MalNEt, while the bacterial DNA polymerase I is resistant (20). Treatment of the extracts of HB101, or of HB101 carrying pATH1, with MalNEt had no effect on the activity; but treatment of extracts of cells carrying pSH1 reduced the high level of activity to that of the control extracts (Table 1). This result suggested that the pSH1 plasmid induced a reverse transcriptase activity with properties similar to those of the authentic enzyme.
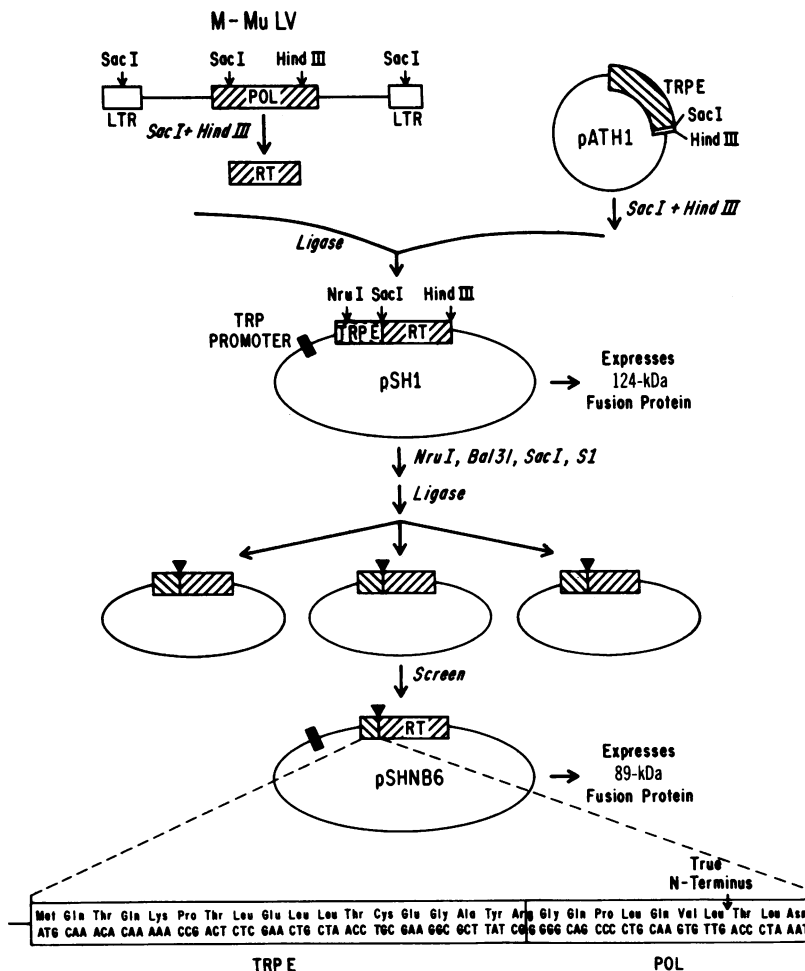

FIG. 1. Construction of trpE–pol gene fusions. The central portion of the pol gene was excised from a cloned copy of the Moloney murine leukemia virus (M-MuLV) genome by cleavage with Sac I and HindIII and inserted into the polylinker sequence of the expression vector pATH1. The resulting plasmid, pSH1, expressed a 124,000-Da fusion protein and substantial levels of active reverse transcriptase. In a second step, the bulk of the trpE sequences and various amounts of the pol gene were deleted, and bacterial clones were screened for increased levels of reverse transcriptase activity. The highest-level producer, pSHNB6, encoded an 89,000-Da protein. The DNA sequence in the region of the deletion, along with the predicted amino acid sequence of the encoded protein, is indicated.
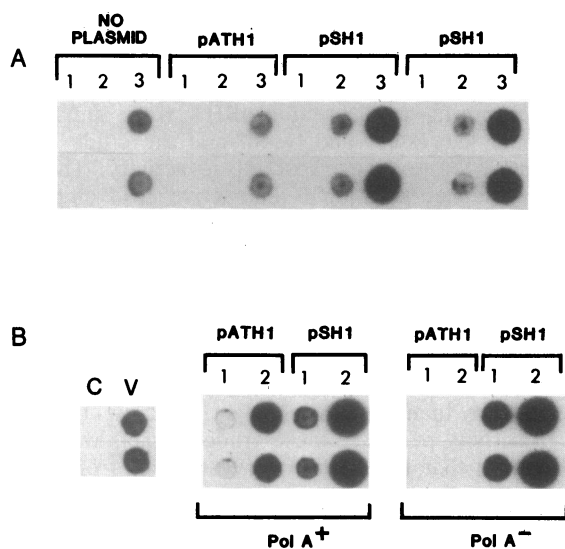
FIG. 2. Reverse transcriptase assays of bacterial extracts of total proteins. Aliquots were incubated in a reaction cocktail containing labeled precursors, and the products were spotted on DEAE paper, washed, and exposed to x-ray film. The two rows show duplicate assays. (*A*) Extracts of HB101 cells carrying the indicated plasmid were prepared and either 0.1 μl (lanes 1), 1 μl (lanes 2), or 10 μl (lanes 3) were assayed. Cells carrying pSH1 expressed a 5- to 10-fold higher level of activity over control cells. (*B*) Lane C, control cocktail alone. Lane V, virus preparation from infected NIH/3T3 cells (10 μl). Set marked Pol A$^+$ represents HB101 cells carrying the indicated plasmids, assayed using 1 μl (lanes 1) or 5 μl (lanes 2) of extract. Set marked Pol A$^-$ represents C2110 cells carrying the indicated plasmids assayed as described.

Further evidence that the additional activity was not due to increased levels of DNA polymerase I was obtained by repeating the assays in a bacterial host carrying a mutation in *polA*, the structural gene for the enzyme. Strain C2110 (*polA1$^-$*) was transformed to ampicillin resistance with pATH1 and pSH1; because the *polA* gene is required for plasmid replication, these transformations occur at low frequency. The plasmids are apparently maintained by recombination with the host chromosome (data not shown). The continued presence of the *polA1$^-$* mutation was confirmed (21) by testing the strains for sensitivity to methyl methanesulfonate. Extracts of strain C2110, or of strain C2110 bearing the pATH1 vector, showed no measurable reverse transcriptase activity, confirming that the background activity of HB101 was indeed due to DNA polymerase I (Fig. 2*B*). Extracts of C2110 bearing pSH1 showed the same high levels of activity seen in the HB101 host (Fig. 2*B*; Table 1). It is noteworthy that the single copy of the gene fusion in the C2110 cells expressed as much activity as the multicopy genes in HB101.

Removal of the *trpE* Sequences Results in Increased Enzyme Activity. Cultures containing each of the variant plasmids generated by mutagenesis of pSH1 were grown and starved for tryptophan, and extracts were assayed for reverse transcriptase activity. Approximately 100 independent clones were screened, and 2 were found to produce dramatically higher levels of activity than the parental pSH1 plasmid (Fig. 3). One of these clones, carrying plasmid pSHNB6, was chosen for further study. Quantitative assays reproducibly showed that cells carrying the new plasmid expressed a 4- to 8-fold higher level of activity than cells carrying pSH1; the cells showed as much as a 35-fold increase in activity over cells carrying the pATH1 vector alone (Table 1).

Sequence analysis of the pSHNB6 DNA (Fig. 1) showed that 18 codons of *trpE* were joined, in the correct reading frame, to the *pol* gene; the remaining portion of the *pol* gene

Table 1. DNA synthesis on synthetic templates by bacterial extracts: Sensitivity of MalNEt

Cells	Plasmid	Treatment	[^{32}P]dTTP incorporated, pmol/μl
		Exp. 1	
HB101	pATH1	Lysate	2.00
		+ MalNEt, then DTT	1.55
		+ DTT, then MalNEt	1.40
	pSH1	Lysate	23.7
		+ MalNEt, then DTT	2.48
		+ DTT, then MalNEt	15.9
		+ premixed DTT/MalNEt	16.9
		Exp. 2	
HB101	pATH1	Lysate	59.6
		+ MalNEt, then DTT	45.7
	pSH1	Lysate	751
		+ MalNEt, then DTT	98.2
	pSHNB6	Lysate	2090
		+ MalNEt, then DTT	237
C2110	pSH1	Lysate	1590
		+ MalNEt, then DTT	164
		Exp. 3	
HB101	pSH1	DEAE eluate	112
		+ MalNEt, then DTT	27.3
		40–70% AS fraction	140
		+ MalNEt, then DTT	94.4
		0–40% AS fraction	252.6
		+ MalNEt, then DTT	25.5

Lysates were prepared from the indicated bacterial cells carrying the indicated plasmids and were assayed for reverse transcriptase after various treatments. Results expressed as pmol of [^{32}P]dTTP incorporated into DNA per μl of extract under standard conditions (see *Methods*). Extracts for Exp. 1 were made by the dilute lysis procedure, those for Exp. 2 were made by the concentrated lysis procedure, and those for Exp. 3 were made as described in the text. Protein concentrations (in mg/ml) were as follows: Exp. 1, 1.34 and 1.51; Exp. 2, 3.82, 3.10, 3.53, and 3.6; Exp. 3, 1.05, 2.39, and 0.59. DTT, dithiothreitol; AS, ammonium sulfate.

contained 7 codons 5' to the start of the mature reverse transcriptase. The plasmid in the second highly active clone, pSHNB63, also retained similar *trpE* coding sequences (data not shown). These results suggest that the presence of the *trpE* codons upstream of this site may help to stabilize the protein product.

Analysis of Fusion Proteins Encoded by pSH1 and pSHNB6. We next analyzed the polypeptides synthesized in HB101 cells carrying the pSH1 and pSHNB6 plasmids. Both the total proteins and the proteins remaining insoluble after addition of detergent and high salt concentrations were isolated (17). These fractions were subjected to NaDodSO$_4$/polyacrylamide gel electrophoresis, and the proteins were detected by Coomassie stain (Fig. 4*A*). The sequence of the pSH1 plasmid predicted the formation of a fusion protein containing both the *trpE* and *pol* polypeptides, of 124,000 Da; cells carrying pSH1 exhibited a major new protein migrating at ≈120,000 Da, in good agreement with the expected size. Virtually all of this polypeptide was recovered in the insoluble fraction. In addition, many smaller proteins specific to cells carrying pSH1 were detected. These proteins, ranging in size from 110,000 to 52,000 Da, represented a major portion of the total mass of the new protein induced by pSH1. We have found that extended incubation of the extracts to allow autolysis did not increase the formation of these smaller proteins and that the addition of protease inhibitors during the lysis did not reduce the formation of these products.

The structure of pSHNB6 plasmid predicted the synthesis of a protein of 89,000 Da; examination of cells carrying

A　　　　　　B　　　　　　C

1　0.1　ul　　　1　0.1　ul　　　1　0.1　ul

pATH1　　　　　pATH1　　　　　pATH1

pSH1　　　　　　pSH1　　　　　　pSH1

　　　　　　　　pSHNB6　　　　　pSHNB6

pSHNB2　　　　　　　　　　　　　pSHNB63

6　　　　　　　51　　　　　　　71

15　　　　　　53　　　　　　　72

16　　　　　　54　　　　　　　85

17　　　　　　55　　　　　　　86

30　　　　　　56　　　　　　　89

37　　　　　　58　　　　　　　90

38　　　　　　62　　　　　　　91

39　　　　　　63　　　　　　　92

40　　　　　　66　　　　　　　93

41　　　　　　67　　　　　　　94

42　　　　　　68　　　　　　　95

43　　　　　　73　　　　　　　96

44　　　　　　74　　　　　　　97

45　　　　　　75　　　　　　　98

46　　　　　　76　　　　　　　99

47　　　　　　77　　　　　　　100

48　　　　　　78　　　　　　　101

C　　　　　　　80　　　　　　　102

V　　　　　　　81　　　　　　　103

　　　　　　　　82　　　　　　　104

　　　　　　　　84
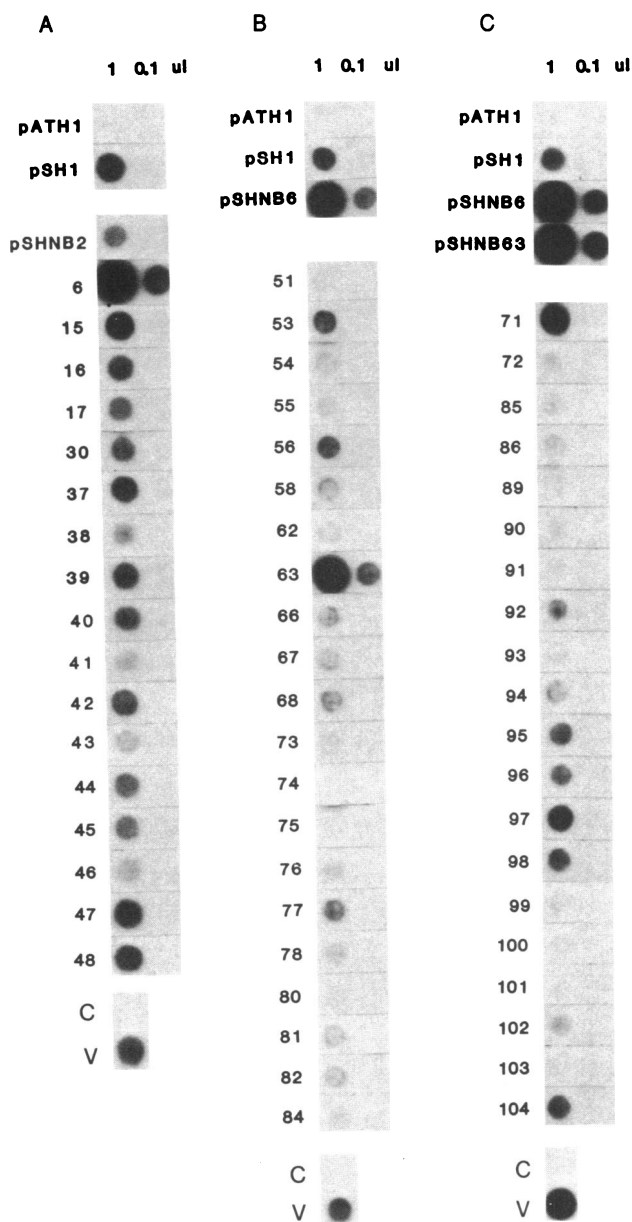
　　　　　　　　C　　　　　　　C

　　　　　　　　V　　　　　　　V

FIG. 3. Reverse transcriptase assays as a screen of cloned variants of the pSH1 plasmid. Indicated amounts of total protein extract from each clone were assayed as described in Fig. 2. Samples C and V are cocktail alone and viral preparations. Cells carrying pATH1 and pSH1 are shown as standards.

pSHNB6 showed substantial amounts of a new protein of ≈90,000 Da, as well as lesser amounts of smaller proteins (Fig. 4B). These proteins could be detected in total lysates, but there were high levels of other bacterial proteins in the same region of the gels. Analysis of the proteins labeled with [^{35}S]methionine at the time of induction of the *trpE* gene clearly revealed the 90,000-Da protein and two major species at ≈70,000 and ≈60,000 Da.

The identity of these proteins as products of the gene fusion was confirmed by immunoprecipitation with specific antisera. Cells were labeled with [^{35}S]methionine after induction of the *trp* operon. The proteins were extracted with detergents, immunoprecipitated, and analyzed by electrophoresis and fluorography (Fig. 4C). Cells carrying pSH1 contained new proteins reactive with the specific sera. A protein of 120,000 Da and a number of smaller polypeptides ranging in size from 110,000 to 60,000 Da were barely detectable with the *trpE* serum (lane 6). A similar spectrum of proteins was readily seen with either of two sera reactive with reverse

transcriptase (lanes 7 and 8), demonstrating that the new proteins contain determinants of both *trpE* and reverse transcriptase. The soluble fractions were highly enriched for the smaller proteins relative to the full-length product, compared with the insoluble fraction, suggesting that the small proteins were selectively extracted.

Immunoprecipitation of the proteins from cells carrying pSHNB6 with sera specific for viral reverse transcriptase showed that a 90,000-Da protein and at least one major smaller protein were also recognized by the sera (Fig. 4C, lanes 11 and 12). A higher proportion of the full-size pSHNB6 protein was recovered in the soluble fraction than with the corresponding pSH1 protein. Thus, the removal of the bulk of the *trpE* sequences, and possibly part of the 5' *pol* sequences, resulted in the synthesis of a smaller protein with increased solubility, improved stability to proteolytic degradation, and exhibiting a higher level of reverse transcriptase activity. We have not determined whether the higher activity is due solely to an increase in the amount of soluble full-size protein recovered, or whether it is also due to an increase in the specific activity of the enzyme.

Partial Purification of the Fusion Proteins. To characterize further the new activities induced by the gene fusions, and to demonstrate that the new DNA synthetic activity was not due to DNA polymerase I, we partially purified the activities. The bulk of the DNA in the solution was removed by precipitation with streptomycin sulfate; 80% of the activity remained soluble through this procedure. The material was applied to a DEAE cellulose column at a low ionic strength, and the activity was recovered by elution with buffer containing 0.2 M NaCl.

Fractionation of the eluate from cells carrying pSH1 by ammonium sulfate precipitation showed (Table 1) that most of the activity was recovered in the fraction precipitated by 40% saturated ammonium sulfate. The bulk (90%) of the activity in this fraction was sensitive to MalNEt, indicating that the activity was due to pSH1 enzyme. The rest of the initial activity was recovered in the 40–70% ammonium sulfate fraction, known to contain DNA polymerase I (22). This activity, as expected, was largely resistant to MalNEt treatment.

The activity induced by pSHNB6 behaved similarly. After elution from DEAE cellulose, the bulk of this activity was precipitated by addition of ammonium sulfate to 45% of saturation. The crude material was 23% resistant to MalNEt. Greater than 90% of the activity that was precipitated by ammonium sulfate was sensitive and was, therefore, enriched for reverse transcriptase-like activity; the activity that remained soluble was now 72% resistant and, therefore, consisted largely of DNA polymerase I. Further purification of the pSHNB6 protein on phosphocellulose showed that the activity could be bound and eluted with buffer containing 0.1–0.2 M NaCl. Preliminary characterization of this activity showed that long DNA products could be synthesized, and that RNase H activity had copurified through these steps. A more detailed description of the purification and characterization of this and similar fusion proteins will be published elsewhere (23).

DISCUSSION

These experiments demonstrate that portions of the *pol* gene of a mammalian retrovirus can be expressed as a gene fusion with the bacterial *trpE* gene. The fusion proteins are sufficiently abundant and stable to be detected after electrophoresis of the total bacterial proteins and are major proteins in an insoluble fraction of the lysate. Our crude estimate is that the *pol*-related products represent ≈1% of the total protein after induction of the *trp* operon. Extracts containing these proteins show reverse transcriptase activity, as assayed by
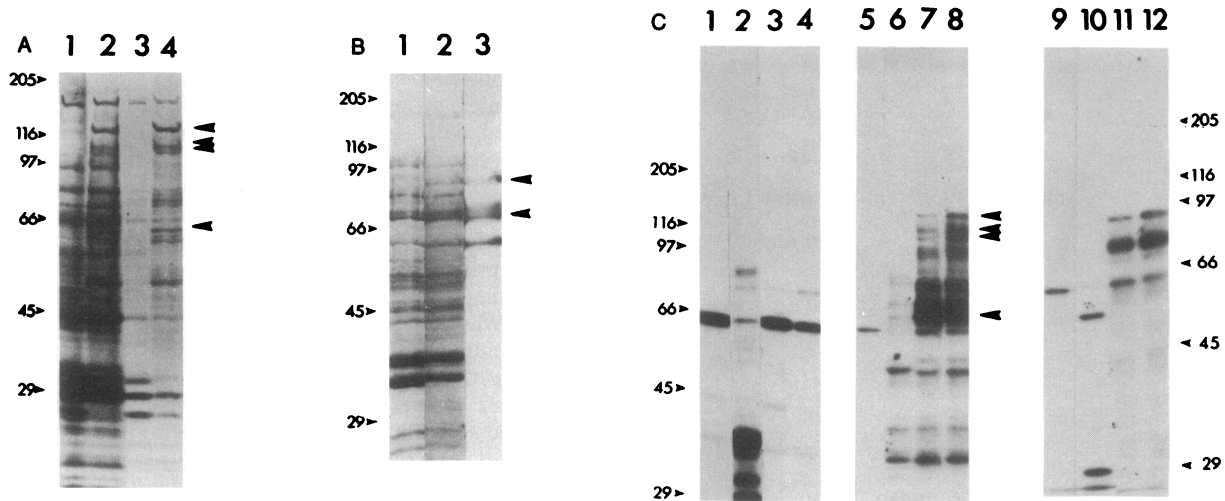
FIG. 4. NaDodSO₄/polyacrylamide gel electrophoresis of bacterial proteins from HB101 cells carrying various plasmids. (*A*) Proteins analyzed by Coomassie blue staining. Lanes: 1, total proteins of cells containing vector pATH1; 2, total proteins of cells containing plasmid pSH1; 3, insoluble fraction from cells carrying pATH1; 4, insoluble fraction from cells carrying pSH1. The major new proteins are indicated by the arrowheads. Positions of the molecular size standards in kDa are indicated. (*B*) Lanes: 1 and 2, Coomassie stain of insoluble proteins; 1, cells without plasmid; 2, cells carrying pSHNB6; 3, fluorogram of proteins labeled after induction of the *trp* operon in cells carrying pSHNB6. Molecular size standards (in kDa) are indicated. Major bands induced by pSHNB6 are visible at 90, 70, and 60 kDa. (*C*) Immunoprecipitation of bacterial proteins. Lanes: 1–4, proteins of control cells carrying the vector pATH1; 5–8, proteins of cells carrying pSH1; 9–12, proteins of cells carrying pSHNB6. Lanes 1, 5, and 9, normal rabbit serum. Lanes 2, 6, and 10, rabbit serum specific for the TrpE protein. Lanes 3, 7, and 11, NCI serum 775-424 specific for reverse transcriptase. Lanes 4, 8, and 12, NCI serum 775-454 specific for reverse transcriptase. Molecular size standards (in kDa) are indicated.

the synthesis of DNA on ribohomopolymer templates. The level of activity is many fold greater than the low activity due to the endogenous DNA polymerase I, is independent of the *polA* gene in the host, and shows biochemical properties distinct from those of this enzyme.

It is clear that the gene constructs lead to the formation of shorter protein products as well, probably formed by degradation of the primary translation product within the cell. The shorter products may be responsible for much of the detectable activity. Thus, modifications in the gene fusion that allow the direct formation of similar smaller proteins might yield higher levels of recoverable activity. The increased activity seen for the pSHNB6 construct is consistent with this notion. Recently, further efforts to trim the size of the gene to its minimum have led to the synthesis of products with increased solubility, stability, and activity (23).

We believe that the expression of the murine reverse transcriptase in bacterial cells will lead to several important projects. Firstly, the availability of large quantities of the purified enzyme will allow extensive characterization of the enzyme. Secondly, mutations can be readily introduced into the cloned gene fusions, and large numbers of bacterial cultures can be screened for the presence of rare variants exhibiting desirable changes in the activity. It may be possible, for example, to construct variants that do not express RNase H activity. Thirdly, mutations such as temperature-sensitive mutations can also be generated, and a DNA fragment containing the alteration can be recovered and reinserted into the complete viral genome. In this way, it may be possible to study the effects of many new mutations on the retroviral life cycle and determine new functions for the reverse transcriptase enzyme.

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