Nucleic Acids Research

High level expression and purification of HhaI methyltransferase

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ABSTRACT

A cloning system for the DNA-(cytosine-5)-methyltransferase MHhaI and high level expression of the enzyme are described. A parent plasmid was constructed from fragments of the MHhaI gene and synthetic oligonucleotides. The construct permits introduction of various restriction sites for cloning at precise positions near the initiation codon, and beyond the termination codon. The entire MHhaI coding sequence was introduced as a 1042 b.p. NdeI-XbaI fragment into the vector pAR3040 which contains the T7 RNA polymerase promoter. The resultant plasmid pTNX3 (MHhaI–pAR3040) was introduced into McrB⁻ E. coli strains HB101 and GM2929, and expression of MHhaI was induced by infection with the λ phage CE6 carrying the T7 RNA polymerase gene. In induced cells, catalytically active MHhaI was produced at a level that corresponds to about 8% of the total soluble protein; an insoluble form of the protein was also formed, but could be readily removed. The expressed soluble enzyme from HB101/pTNX3 was purified to apparent homogeneity in about 50% yield by a two-step chromatographic procedure involving DEAE-cellulose and Heparin-Sepharose; a one liter culture gave about 2.5 mg of pure enzyme. The molecular weight and kinetic properties of the expressed protein are identical to those reported for the authentic MHhaI, and its amino terminal sequence agrees with that predicted from the DNA sequence.

INTRODUCTION

HhaI methylase (MHhaI) is a DNA-(cytosine-5)-methyltransferase (EC 2.1.1.37) from the bacterium *Haemophilus haemolyticus*. The enzyme catalyzes the transfer of a methyl group from S-adenosylmethionine (AdoMet) to C5 of the internal cytosine in the tetranucleotide DNA sequence GCGC (1). The gene for MHhaI has been sequenced (2), and the kinetics and catalytic properties of the enzyme have been investigated (3). This laboratory is studying the structure and function of DNA-(cytosine-5)-methyltransferases and related enzymes that methylate pyrimidines at the 5-position (3,4,5). We have shown that methylation proceeds by an ordered mechanism: DNA binds first and departs last from the en-

zyme. The catalytic mechanism appears to be analogous to that of dTMP synthase; it involves initial formation of a covalent adduct between an enzymic nucleophile and the 6-carbon of cytosine to activate the 5-position for one carbon transfer.

More extensive studies of the structure and function of MHhaI require reagent quantities of enzyme and a convenient method of purification. The best current source of MHhaI is plasmid pHhaI, which expresses the gene in *E. coli* under control of its natural promoter (2). However, production by pHhaI is insufficient for studies that require large amounts of enzyme. In this work, we describe cloning of the MHhaI coding sequence under control of the T7 RNA polymerase promoter and high expression of catalytically active enzyme in *E. coli*. We also describe an efficient two step purification and properties of the expressed MHhaI.

MATERIALS AND METHODS

AdoMet (p-tosyl salt) and AdoHcy were purchased from Sigma. Poly(dG-[5-^{3}H]dC) was prepared as previously described (3). Oligonucleotides were synthesized at the Biomolecular Resource Center of UCSF (BRC). Restriction enzymes and commercial MHhaI was purchased from New England Biolabs. Heparin-Sepharose was from Pharmacia. DE52 was obtained from Whatman. Reverse phase HPLC and amino acid sequence analysis were performed at the BRC.

Bacterial strains, plasmids, phage, and culture conditions

The *Escherichia coli* strain RR1 containing the plasmid pHhaI was a gift of G. Wilson (N. E. Biolabs). Other *E. coli* strains used were HB101, GM2929 (F⁻, *dam*⁻13, *Tn*9, *dcm*⁻6, *hsd*R2, *rec*F143) (obtained from M. Marinus), ED8739 (F⁻, *met*B, *hsd*S, *sup*E, *sup*F), HMS174 (F⁻, *hsd*R, *rec*A, *Rif*ᴿ), and BL21 (F⁻, *hsd*S, *gal*). ED8739, HMS174, BL21, and the λ phage CE6 which carries T7 gene 1 were obtained from F.W. Studier (6). Plasmids used were pKGS (7), pEMBL18 (8), and pAR3040 (6). Cells and phage cultures were grown in LB medium. When needed, antibiotics were added to media at 50μg/ml kanamycin, or 100μg/ml ampicillin.

Plasmid constructions

Restriction enzyme reactions and ligations were performed in conditions recommended by the supplier of enzymes (N.E. Biolabs). DNA manipulations, transformations, and bacteriophage cultivation were performed using methods described by Maniatis *et. al.* (9).

Assay for MHhaI activity

The methylation activity of MHhaI was measured in two ways. The first method, used in routine assays of enzyme activity, measured the release of 5-^{3}H from poly(dG-[5-^{3}H]dC) (specific activity 200-550 dpm/pmol cytosine) in the presence of saturating concentrations of copolymer (0.25-1.0 μM in ds-recognition sites) and AdoMet (100 μM). The second method employed poly(dG-[^{14}C]dC) as substrate in the presence of [methyl-^{3}H]AdoMet; the extent of reaction was determined by trapping ^{3}H/^{14}C radioactivity with DE52. Reaction conditions, the charcoal assay to measure 5-^{3}H release, and the DE52 elution assay have been previously described (3). Proteins were assayed by the method of Bradford (10).

Purification of MHhaI from HB101/pTNX3 cells

MHhaI was purified from HB101/pTNX3 cells after induction with CE6. One liter of LB-ampicillin containing 0.2% maltose was inoculated with a 30 ml overnight culture of HB101/pTNX3 cells grown in the same medium and incubated at 37°C. At A_{600} = 1.1 (~4 x 10^7 cells per ml, ~3 h), 25 ml of CE6 (5 x 10^{11} pfu) was added and the culture allowed to grow for an additional 3h at 37°C. The cells were collected by centrifugation and the pellet (2.7 g wet weight) was resuspended in 35 ml lysis buffer (10 mM KHPO$_4$, pH 7.4, 10 mM β-mercaptoethanol, 0.1 mM EDTA). Cells were lysed by French Press (~14000 psi, 2 passes), and the suspension was centrifuged at 10,000 x g for 30 min. The supernatant was collected (30ml) and the enzyme was purified by chromatography on DE52 and Heparin-Sepharose (see Figure 5 and Results).

RESULTS

Strategy for construction of overexpressing plasmids

Our objective was to clone the 981 b.p. coding region of MHhaI into a parent plasmid that would permit facile mobilization of the gene into precise positions of expression vectors. Our source of the gene, plasmid pHhaI (Figure 1), contains MHhaI within a 1470 b.p. HindIII fragment and is not well suited for this purpose. This plasmid does not have appropriate unique 5'- or 3'- cloning sites, and unique restriction sites are many bases away from the initiation codon (2). Our strategy for producing the desired parent plasmid was to reconstruct most of the coding sequence with the adjacent 186 b.p. AluI-BclI (Sau3AI) and 825

HindIII   AluI   BclI(Sau3AI)   HindIII

-436   1 23   210(211)   982   1035

MHhaI

ATG   TAA
(Start)   (Stop)
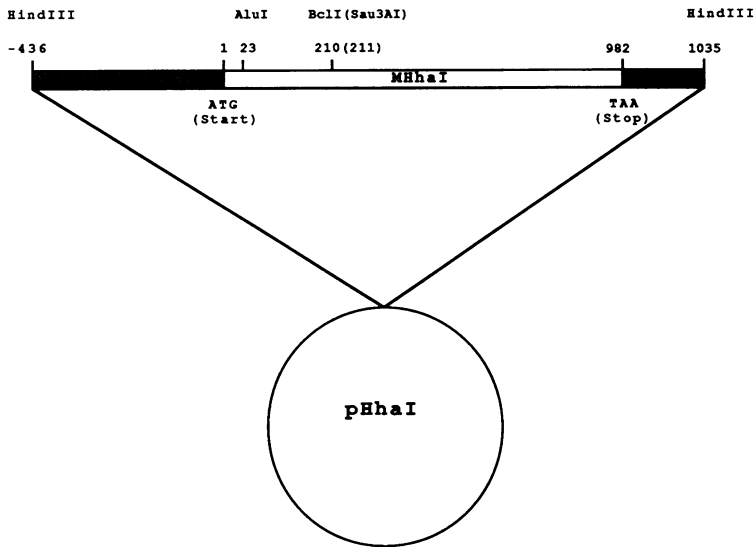
pHhaI

Figure 1. A partial restriction map of the gene for MHhaI located on the plasmid pHhaI. pHhaI is pBR322 in which the MHhaI gene was inserted via the HindIII site. The 981 b. p. coding sequence has an AluI site at positions 23 between the start codon and a unique BclI site at position 210. Restriction by BclI and Sau3AI both generate 5' "sticky" ends with the sequence GATC. The open bar indicates the coding sequence for MHhaI; the shaded regions indicate the 5'- *H. haemolyticus* promoter region and 3'-termination sequence of the gene.

b.p. BclI-HindIII fragments of pHhaI. The 5'-end was constructed from a synthetic adapter containing codons 3 to 8 of the coding sequence and restriction sites which permit introduction of oligonucleotides possessing desired cloning sites (Figure 2A).

Plasmid constructions

The pHhaI plasmid was first introduced and propagated in the dam⁻ *E. coli* strain GM2929 because our strategy required an unmethylated BclI site. The 825 b.p. 3'-terminal BclI-HindIII fragment of the MHhaI gene was excised from pHhaI and purified. The 5'-AluI-Sau3AI 186 b.p. fragment was isolated and purified from pHhaI prepared from the dam⁺ RR1/pHhaI cells.

The XhoI linker (Figure 2A) was inserted into the HindIII-PstI sites of plasmid pKGS to give pKGX (Figure 3). The pKGS plasmid lacks a PvuII site so the only PvuII site of pKGX is in the linker. The unmethylated pKGX obtained from dam⁻ GM2929 cells

A

BamHI XhoI　　　　　　PvuII BclI HindIII XbaI

5'-AGCTGGATCCCTCGAGATAAAAGATAAACAGCTGTGATCAAAGCTTTCTAGACTGCA-3'
3'-　　CCTAGGGAGCTCTATTTTCTATTTGTCGACACTAGTTTCGAAAGATCTG　　-5'

"HindIII"　　　*GluIleLysAspLysGln*
　　　　　　　　3　4　5　6　7　8　　　　　　　　　　　PstI

B

KpnI　　　　　　BspMI　NdeI

5'-　　　　CCGGGGATCCACCTGCGGCATATGA　　-3'
3'- CATGGGCCCCTAGGTGGACGCCGTATACTAGCT -5'

BamHI　　　　　　　　　"XhoI"

C

　　　　　　　1　2　3　4　5　6　7　8　9　10
　　　　　　Met Ile Glu Ile Lys Asp Lys Gln Leu Thr

pHhaI : 5'- ... CTT ATG ATT GAA ATA AAA GAT AAA CAG CTC ACA ... -3'

pKGX : 5'- ... GGA TCC C⌐TC GAG ATA AAA GAT AAA CAG⌐ CTG TGA ... -3'

pEBX1 : 5'- ... GGA TCC C⌐TC GAG ATA AAA GAT AAA CAG⌐ CTC ACA ... -3'

pTNX3 : 5'- ... CAT ATG A⌐TC GAG ATA AAA GAT AAA CAG⌐ CTC ACA ... -3'

Figure 2. The sequences of the oligonucleotides used in plasmid constructions and codon usage in the plasmids. **A)** The DNA sequence of the synthetic oligonucleotide XhoI linker. The 5'-end has a 4 base single strand overhang complementary to HindIII restricted sites; upon ligation to pKGS at the HindIII and PstI sites, the original HindIII site is lost, and the internal HindIII site of the linker becomes unique on the plasmid. The XhoI to PvuII region contains the coding sequence for amino acids 3 to 8 of MHhaI as indicated. **B)** Sequence of the synthetic olignucleotide NdeI linker. This fragment can be ligated to pEBX1 via the KpnI site in the polylinker region and the XhoI site introduced by the XhoI linker (A). Insertion of this linker into pEBX1 restores the MHhaI gene sequence, starting at the ATG of the NdeI site. Additional sites (BspMI and BamHI) were included for diagnostic purposes during plasmid construction. **C)** Codon usage in selected plasmids used in this study. The first ten amino-terminal amino acids of MHhaI and the nucleotide sequence encoding them are shown for pHhaI and pTNX3. The corresponding sequences in the intermediate plasmids pKGX and pEBX1 are also depicted. The boxed nucleotides indicate the coding sequence introduced by the XhoI linker (A). The synonymous codon changes in the final plasmid pTNX3 occur at amino acid positions 2 and 3 (Ile and Glu), in which the ATT and GAA of pHhaI were changed to ATC and GAG,

respectively, of pTNX3. A codon change also occurs at amino acid
position 9 (Leu) in pKGX, but the original codon is restored in
subsequent plasmids after restriction with PvuII (amino acid
postions 8 and 9, CAGCTG) and ligation with the AluI-Sau3AI
fragment (see Figures 1 and 3). The XhoI site of pKGX and pEBX1
(amino acid positions 2 and 3, CTCGAG) is lost upon insertion of
the NdeI linker (B) into pTNX3.

contains the following features relevant to the construction of

the parent cloning plasmid: a) PvuII and BclI sites to accept the

186 b.p. AluI-Sau3AI fragment of pHhaI; b) BclI-HindIII sites for

the insertion of the 825 b.p. BclI-HindIII fragment of the coding

sequence; c) An XbaI restriction site 3'- to the termination se-

quence as an additional 3'- cloning site; d) Restriction sites for

modifying the 5'- end to any desired sequence; and e) nucleotides

5-24 of the coding sequence.

   The remaining strategy is outlined in Figure 3. The 186 b.p.

AluI-Sau3AI fragment was inserted into pKGX via the PvuII-BclI

sites, and the resultant plasmid pKGB2 was grown in GM2929 cells.

The 825 b.p. BclI-HindIII fragment from pHhaI was introduced into

the BclI-HindIII sites adjacent to the 186 b.p. AluI-Sau3AI insert

of pKGB2, and plasmid pKBH4 was isolated. Plasmid pKBH4 contains

all of the coding sequence of MHhaI except the initiation and sec-

ond codons, and does not possess 5'- cloning sites appropriate for

the expression vectors we wished to use. We planned subsequent

manipulations which use the XhoI site introduced in the original

synthetic linker. Since pKGX also possesses a XhoI site originat-

ing from the plasmid pKGS, the 1043 BamHI-XbaI fragment from pKBH4

was moved into pEMBL18, which has no XhoI sites (8). Also, since

there was no further need to maintain the BclI site, the host

cell used for subsequent transformations was the dam[+] HB101.

Plasmid pEBX1 is the parent cloning plasmid into which the first

two codons of the gene for MHhaI as well as other useful upstream

restriction sites can be inserted via the XhoI site and another

site 5'- to it (e.g., KpnI). To construct the vector used in the

T7 RNA polymerase system, an adapter (Figure 2B) containing the

initiation ATG in an NdeI site was introduced via the KpnI-XhoI

sites of the plasmid to generate pEN1. Excision of the 1042 b.p.
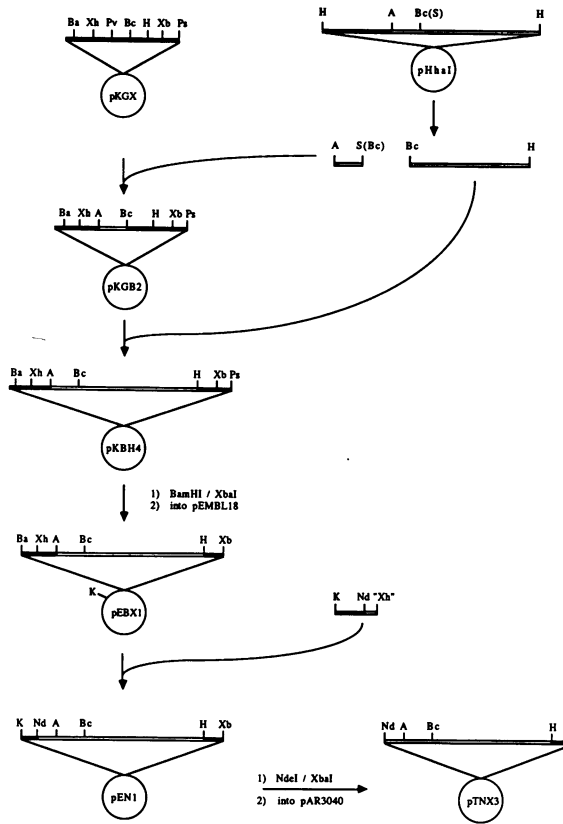
Figure 3. The strategy for the construction of plasmids. The XhoI adapter (Figure 2A) is first inserted into pKGS to give the plasmid pKGX. Insertion of the 186 b.p. AluI-Sau3AI fragment of the MHhaI gene (positions 25-210) into pKGX via the PvuII and BclI sites of the adapter yields the plasmid pKGB2. Insertion of the 825 b.p. BclI-HindIII fragment of the MHhaI gene (positions 211-1035) reconstitutes the gene from position 7-1035 in the plasmid pKBH4. The 1043 b.p. BamHI-XbaI fragment of pKBH4 is then inserted into pEMBL18 via the BamHI and XbaI sites in the polylinker region to give the plasmid pEBX1 Insertion of the NdeI linker (Figure 2B) restores the complete coding sequence of MHhaI in pEN1. Excision of the gene sequence as a 1042 b.p. NdeI-XbaI fragment and insertion into pAR3040 via the NdeI and NheI sites gives the overproducing plasmid pTNX3. Abbreviations for the restriction enzymes are: A, AluI; Ba, BamHI; Bc, BclI; H, HindIII; K, KpnI; Nd, NdeI; Ps, PstI; Pv, PvuII; S, Sau3AI; Xb, XbaI; Xh, XhoI.

NdeI-XbaI fragment from pEN1 and ligation to the NdeI and NheI sites of pAR3040 yielded plasmid pTNX3, which contains the complete gene for MHhaI located downstream from the T7 promoter.

Expression of MHhaI from HB101/pEN1

Although the pEN1 plasmid was not the ultimate goal of these constructions, the MHhaI gene was expressed in HB101/pEN1 as determined by assays of enzyme activity in cell extracts (~10 units/mg protein) and by resistance of the plasmid to restriction by HhaI endonuclease. Since the gene is located downstream from the *lac* promoter in pEMBL18, its activity is also potentially inducible by IPTG. Although the coding sequence is not in frame with that of the β-galactosidase sequence in this plasmid and the HB101 host does not contain the lacIQ' gene, induction increases the MHhaI activity in cell lysates to approximately five-fold that of the uninduced state (specific activity ~46 units/mg protein).

Expression of MHhaI from pTNX3 in GM2929 and HB101

The pTNX3 plasmid isolated from HB101/pTNX3 was introduced into GM2929 cells, and expression of MHhaI was measured following infection by CE6. Expression was not initially attempted in HB101 cells because they are *supE* which should lyse upon infection by λ phage. With increasing CE6, the level of MHhaI in GM2929/pTNX3 cells increases to about 2500 units/mg at m.o.i. ~50 (Figure 4). At m.o.i. 168, MHhaI activity (~2900 units/mg protein) in soluble fractions is nearly 1000-fold that of the uninduced state (~3 units/mg protein). This activity is about ten-fold that of the parent system RR1/pHhaI (~350 units/mg protein). Calculation of the amount of MHhaI in the soluble pool based on the k_{cat} (1.3 min^{-1}) for this enzyme (3) indicates that native MHhaI constitutes about 3% of the total soluble protein in maximally induced cells, compared to <0.4% in RR1/pHhaI cells. Analysis of the cell lysates by SDS-PAGE reveals a protein band which migrates with the same mobility as authentic MHhaI, with an intensity in proportion to the activity profile (data not shown).

The m.o.i. (>100, Figure 4) required to achieve optimal expression in GM2929 cells is ten-fold greater than that reported for other cells used in the T7 RNA polymerase system (6). This indicated that GM2929 may not be the ideal host for expression by CE6 infection, and we investigated *E. coli* HB101 as a potential host. Figure 4 shows the effect of CE6 infection upon expression of MHhaI in HB101/pTNX3 cells. The highest specific activity of the enzyme in the crude lysate from HB101 cells is about three-
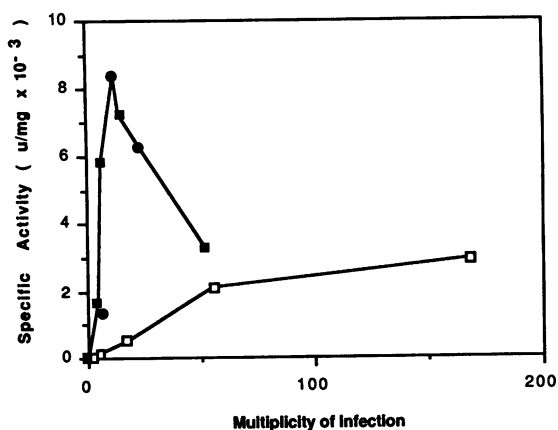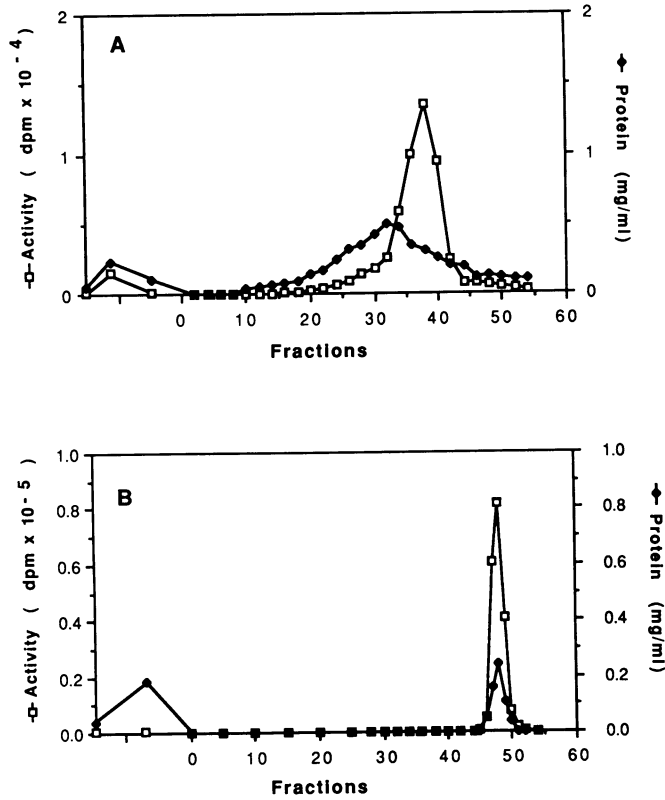
Figure 4. Expression of MHhaI from HB101/pTNX3 and GM2929/pTNX3 as a function of CE6 multiplicity. 25 ml cultures of HB101/pTNX3 and GM2929/pTNX3 cells were grown from a 1/50 dilution of overnight inocula. At cell densities ~2 x 10^7/ml, CE6 was added. After additional incubation for 3 h at 37°C, the cells were collected, lysed, and the lysate cleared by centrifugation at 12,000 x g for 20 min. The lysates were assayed for total protein and for MHhaI activity by the 5-^{3}H exchange procedure as described in methods. ■,●: HB101/pTNX3 (two experiments); □ : GM2929/pTNX3.

fold that of the activity in GM2929 cells. In the induction leading to the highest specific activity (m.o.i. = 14), the amount of soluble enzyme (>7000 units/mg protein) represents about 8% of the soluble proteins based on densitometry of SDS-PAGE gels or on the specific activity of purified enzyme relative to that in the lysate. Further, the optimal m.o.i. of ~14 is in agreement with the values reported by Studier and Moffatt (6). At higher multiplicities the yield and specific activity of MHhaI decreases, as overall protein synthesis in the cells is inhibited (6).

A significant fraction (~30% of pelleted proteins) of the total MHhaI expressed in these cells is found in the lysate pellet (data not shown). The inducible protein in the pellet migrates on SDS-PAGE with the same mobility as the enzyme from the soluble pool. There is, however, no detectable MHhaI activity in resuspended pellets (data not shown).
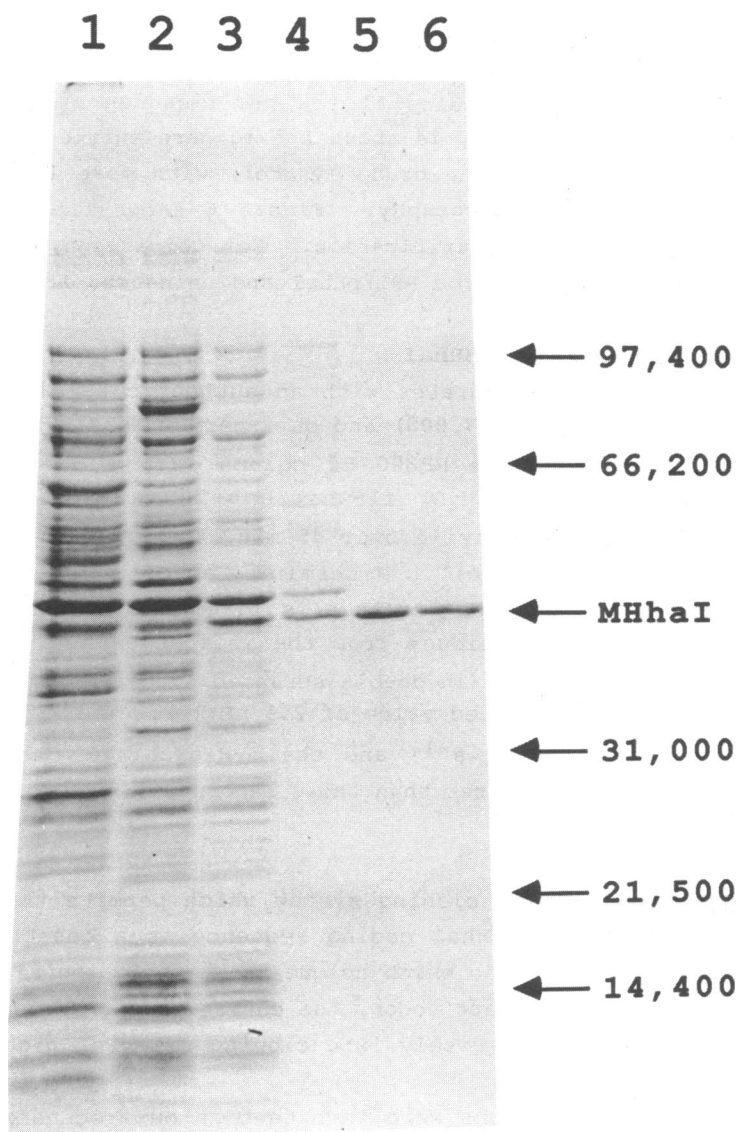
Purification of induced MHhaI from HB101/pTNX3 cells

The purification of MHhaI from a one liter culture of induced HB101/pTNX3 cells by DE52 and Heparin-Sepharose chromatography is

Figure 5. Purification of MHhaI from HB101/pTNX3. Enzyme activity was measured by the 5-^{3}H release procedure as described in methods. A) DE52 column chromatography. Supernatant from centrifuged crude lysate (30 ml) was loaded onto a 2.5 x 10 cm DE52 column equilibrated in lysis buffer (flow rate = 0.5 ml/min). A 400ml linear gradient of 0-0.4M NaCl in lysis buffer was applied and 7 ml fractions were collected. Active fractions (34-42) were pooled. B) Heparin-Sepharose column chromatography of DE52 pool. The DE52 pool (63 ml) was applied to a 2.5 x 10 cm Heparin-Sepharose column equilibrated in lysis buffer. A linear NaCl gradient (400ml, 0-1.2M) in lysis buffer was applied to the column at a flow rate of 0.32 ml/min. Active enzyme eluted as a homogeneous protein and was pooled (fractions 48-49 = pool 1, fraction 47 = pool 2), and dialyzed against 20 mM TrisHCl, pH 8.0, 50 mM NaCl, 0.1 mM EDTA, 10 mM β-mercaptoethanol. The dialyzed enzyme (14 ml Pool 1, 7.5 ml Pool 2) was made 50% in glycerol and stored at -20°C.

shown in Figure 5. The procedure takes advantage of DE52 chromatography as a first step to remove nucleic acids and to effect a 3.6-fold purification. Further, since MHhaI elutes from DE52 at

Figure 6. SDS-PAGE (10-15% gradient gel stained with Coomassie R-250) of MHhaI from cleared lysates of RR1/pHhaI, uninduced HB101/pTNX3, and from CE6-induced HB101/pTNX3 at different stages of purification. lane 1: lysate from RR1/pHhaI cells; lane 2: lysate from uninduced HB101/pTNX3 cells; lane 3: lysate from induced HB101/pTNX3 cells (m.o.i. = 12); lane 4: DE52 pool; lane 5: Heparin-Sepharose pool 1; lane 6: Heparin-Sepharose pool 2. The molecular weights of standards and the position of the MHhaI bands are indicated by arrows.

~0.20 M and from Heparin-Sepharose at ~0.53 M, the eluted protein from the DE52 column can be applied directly to Heparin-Sepharose. Table I summarizes the results of the purification steps. The overall recovery of activity is about 2.5 mg pure enzyme per liter culture (~50% yield from the crude lysate), with most losses occurring upon DE52 chromatography. Figure 6 shows the SDS-PAGE profiles of each step of purification. For comparison, the protein profiles of lysates from RR1/pHhaI and uninduced HB101/pTNX3 cells are also shown.

Properties of purified MHhaI

Purified MHhaI co-migrates with an authentic sample of the enzyme on SDS-PAGE (M_r = 37,000) and on a narrow-bore HPLC system using a Brownlee Aquapore RP300 C8 column (2.1 x 30 mm, 0.1% trifluoroacetic acid in H_2O, flow rate = 200 µl/min); with a gradient of 0-70% acetonitrile over 45 min, the enzyme elutes at 30 min (47% acetonitrile). N-terminal amino acid sequence analysis indicated that the sequence is identical to the deduced sequence (2) up to 18 residues from the initiation Met. The K_m for poly(dG-dC) is 2.3 nM (in double-stranded sites), in agreement with the previously reported value of 2.1 nM (3). The k_{cat} values of the methylation (0.08 s^{-1}) and the 5-H-exchange (0.41 s^{-1}) reactions are ~4-fold greater than those reported (3).

DISCUSSION

We have developed a cloning system which permits the mobilization of the entire MHhaI coding sequence as a casette. By constructing a plasmid into which unique 5'- restriction sites can be introduced near the start codon, the entire coding sequence may be excised and placed precisely into cloning sites of high level expression vectors.

Several considerations were important in our reconstruction of the MHhaI sequence. First, there are methylcytosine-specific nucleases present in many strains of *E. coli* (11); methylated plasmids or those that direct the expression of DNA-cytosine-5-methyltransferases such as MHhaI cannot be propagated in these cells (2,11,12,13). To avoid potential problems until the final stage of the construction, we masked the initiation codon in cloning plasmids by replacing it with an oligonucleotide adapter.

Second, there is a paucity of unique restriction sites near the 5'- initiation codon and within the MHhaI coding sequence, but a unique BclI site occurs at position 210 of the sequence. There is also a single AluI site (position 23) between the start codon and the BclI site; thus all but the first 8 codons of the coding sequence are contained in the adjacent AluI-BclI (positions 25-210) and BclI-HindIII (positions 211-1035) fragments.

Our general approach was to construct a parent plasmid (pEBX1) in which desired restriction sites could be introduced near the initiation codon of the MHhaI gene via insertion of an appropriate oligonucleotide (Figure 2B). To do this, an adapter (Figure 2A) having the following features was introduced into pKGS: a) restriction sites (PvuII, BclI, and HindIII) for insertion of the AluI-BclI and BclI-HindIII fragments of the MHhaI gene, b) unique 5'- BamHI and 3'- XbaI cloning sites, c) the second and third nucleotides of codon 2 and codons 3-8, d) a unique XhoI site at nucleotides 4 to 9 of the coding sequence.

After introduction of the AluI-BclI and BclI-HindIII fragments into pKGX to give pKBH4, the 1043 b.p. BamHI-XbaI fragment was transferred into pEMBL18 to generate pEBX1 (Figure 3). This transfer renders the XhoI site in the linker of pEBX1 unique. Cleavage at the KpnI-XhoI sites permits introduction of synthetic oligonucleotides of the general structure 5'-KpnI-X_n-ATGA-3'/3'-KpnI-X_n-TACTAGCT-5'. Such oligonucleotides provide the initiation codon (underlined), the first base of the second codon, the XhoI overhang, and convenient 5'-cloning sites ($-X_n-$). Insertion of the KpnI-NdeI-XhoI adapter (Figure 2B) into pEBX1 produced plasmid pEN1 containing the entire MHhaI coding sequence with an NdeI site at the start codon and termination sequences at the 3'-end (2). Insertion of the 1042 b.p. NdeI-XbaI fragment into pAR3040 yielded the plasmid pTNX3, in which the coding sequence is positioned under the control of the T7 RNA polymerase promoter.

The expression system using T7 RNA polymerase-directed protein synthesis normally uses HMS174 or BL21(DE3) *E. coli* cells (6). We were unable to transform these cells with pTNX3, probably because of the 5-methylcytosine-specific nucleases in these strains of *E. coli* (11). We used *E. coli* strains HB101 and GM2929 for expression because they lack the McrB activity, which degrades

DNA containing $G^{me}C$ sequences generated by MHhaI (11).

The maximal level of MHhaI activity obtained from GM2929/pTNX3 cells following CE6 induction is greater than 1000-fold the level of protein found in uninduced cells (Figure 4). However, the high m.o.i. (>100) required to achieve high expression in these cells prompted us to examine expression in HB101. HB101/pTNX3 cells express MHhaI optimally at a much lower m.o.i. of ~14. Enzyme activity is increased 2000-fold upon maximal induction (Figure 4), and is about fourteen-fold higher than that obtained from the parent plasmid pHhaI in RR1 cells. Much of the expressed MHhaI in the induced cells is in an inactive precipitated form (30% of pelleted proteins), but the yield of the active enzyme in the soluble fraction of cell lysates is high, representing 8% of total souble proteins in induced HB101 cells.

A two-step purification by DE52 and Heparin-Sepharose chromatography gave homogeneous MHhaI in 52% yield (2.4 mg from a one liter preparation). The purified enzyme co-migrated with authentic MHhaI on SDS-PAGE and reverse phase HPLC. The N-terminal amino acid sequence (from Met1 to Phe18) corresponds to that deduced from the DNA sequence (2). Since the yield of Met1 was 95% based on the amount of protein analyzed, the expressed protein appears to be deformylated at the N-terminus. The protein sequence spans the modifications introduced by the XhoI and NdeI adapters, and verifies that they are expressed without anomaly.

The enzyme has a k_{cat} of 0.08 sec^{-1} for the AdoMet-dependent methyltransferase activity and a k_{cat} of 0.41 sec^{-1} for the AdoMet-independent 5-H exchange of poly(dG-[5-^{3}H]dC) (3). The ratio of exchange to methylation activities is 5, in reasonable agreement with the previously determined value of 7 (3). The k_{cat} values are about three to four times greater than previously reported; we believe these represent better estimates because they were determined for a homogeneous preparation of freshly purified enzyme. The K_m (2.3 nM in double-stranded sites) for poly[dG-dC] agrees with the previous value (2.1 nM) (3). The k_{cat}/K_m for the exchange reaction can now be revised upwards to 1.9 x 10^8 $M^{-1}s^{-1}$ from the previously calculated value of 6.7 x 10^7 $M^{-1}s^{-1}$ (3), supporting our proposal that the MHhaI-catalyzed exchange reaction proceeds at a diffusion-controlled rate.

With the availability of large amounts of MHhaI, we are looking towards studies which will definitively identify the active-site nucleophile of this enzyme (3), and will address issues of sequence recognition and processivity. The availability of the clones described here will also facilitate studies on site-directed mutagenesis.

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