Glucuronyltransferase Activity of KfiC from Escherichia coli Strain K5 Requires Association of KfiA: KfiC AND KfiA ARE ESSENTIAL ENZYMES FOR PRODUCTION OF K5 POLYSACCHARIDE, N-ACETYLHEPAROSAN

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*KfiC AND KfiA ARE ESSENTIAL ENZYMES FOR PRODUCTION OF K5 POLYSACCHARIDE, N-ACETYLHEPAROSAN**

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Heparan sulfate is a ubiquitous glycosaminoglycan in the extracellular matrix of most animals. It interacts with various molecules and exhibits important biological functions. K5 antigen produced by *Escherichia coli* strain K5 is a linear polysaccharide *N*-acetylheparosan consisting of GlcUA β1–4 and GlcNAc α1–4 repeating disaccharide, which forms the backbone of heparan sulfate. Region 2, located in the center of the K5-specific gene cluster, encodes four proteins, KfiA, KfiB, KfiC, and KfiD, for the biosynthesis of the K5 polysaccharide. Here, we expressed and purified the recombinant KfiA and KfiC proteins and then characterized these enzymes. Whereas the recombinant KfiC alone exhibited no GlcUA transferase activity, it did exhibit GlcUA transferase and polymerization activities in the presence of KfiA. In contrast, KfiA had GlcNAc transferase activity itself, which was unaffected by the presence of KfiC. The GlcNAc and GlcUA transferase activities were analyzed with various truncated and point mutants of KfiA and KfiC. The point mutants replacing aspartic acid of a D*X*D motif and lysine and glutamic acid of an ionic amino acid cluster, and the truncated mutants deleting the C-terminal and N-terminal sites, revealed the essential regions for GlcNAc and GlcUA transferase activity of KfiC and KfiA, respectively. The interaction of KfiC with KfiA is necessary for the GlcUA transferase activity of KfiC but not for the enzyme activity of KfiA. Together, these results indicate that the complex of KfiA and KfiC has polymerase activity to synthesize *N*-acetylheparosan, providing a useful tool toward bioengineering of defined heparan sulfate chains.

Heparan sulfate (HS)[3] is a linear polysaccharide of alternating hexuronic acid (D-glucuronic acid (GlcUA) or L-iduronic acid (IdoUA)) and D-glucosamine (GlcN) residues carrying sulfogroups at various sites of sugar residues. Usually, HS chains are covalently attached to a core protein in the form of proteoglycans and are present ubiquitously on the cell surface and in the extracellular matrices of animals. HS chains interact with cytokines, growth factors, coagulation factors, proteases and their inhibitors, and other molecules and contribute to several biological processes, including development, morphogenesis, cell proliferation and differentiation, and cancer cell invasion (1, 2).

The biosynthesis of HS begins with the synthesis of the linkage tetrasaccharide, GlcUA-Gal-Gal-Xyl, on the serine residues of core proteins. The backbone of HS polysaccharide is then synthesized onto the linkage tetrasaccharide by alternating addition of monosaccharide units of GlcNAc and GlcUA with α1–4 and β1–4 bonds, respectively, using UDP-sugar donors with desired GlcNAc transferase (GlcNAc-T) and GlcUA transferase (GlcA-T). The elongation reaction is performed by HS co-polymerases, EXT1 and EXT2, in the Golgi apparatus (3, 4). The backbone is modified by several reactions, including *N*-deacetylation and *N*-sulfonation of GlcN by *N*-deacetylase/*N*-sulfotransferase, C5-epimerization of GlcUA to form IdoUA by HS C5-epimerase, 2-*O*-sulfation of IdoUA and GlcUA by HS 2-*O*-sulfotransferase, 6-*O*-sulfation of GlcN by HS 6-*O*-sulfotransferases, and 3-*O*-sulfation of GlcN by HS 3-*O*-sulfotransferases. These modifications yield HS chains with a variety of structures, providing the specific functions of HS (5, 6).

Capsular polysaccharides are a Gram-negative bacteria coat on the outer membrane of bacterial cells. They play important roles in virulence and survival to protect against nonspecific host defense (7). K5 antigen produced by *Escherichia coli* strain K5 is a linear capsular polysaccharide consisting of GlcUA β1–4 and GlcNAc α1–4 repeating disaccharide, which is an analogue of the unsulfated and unepimerized HS backbone (*N*-acetylheparosan, HPR) (8). The HPR chain of K5 antigen is synthesized onto a phospholipid initiator by the alternate addition of the two monosaccharides on the cytoplasmic surface of the plasma membrane (9). The nascent polysaccharide moves across the plasma membrane, periplasmic space, and the outer membrane and then forms the capsule on the extracellular layer.

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[3] The abbreviations used are: HS, heparan sulfate; GlcA-T, GlcUA transferase; GlcNAc-T, GlcNAc transferase; HPR, *N*-acetylheparosan; TF, trigger factor; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol.

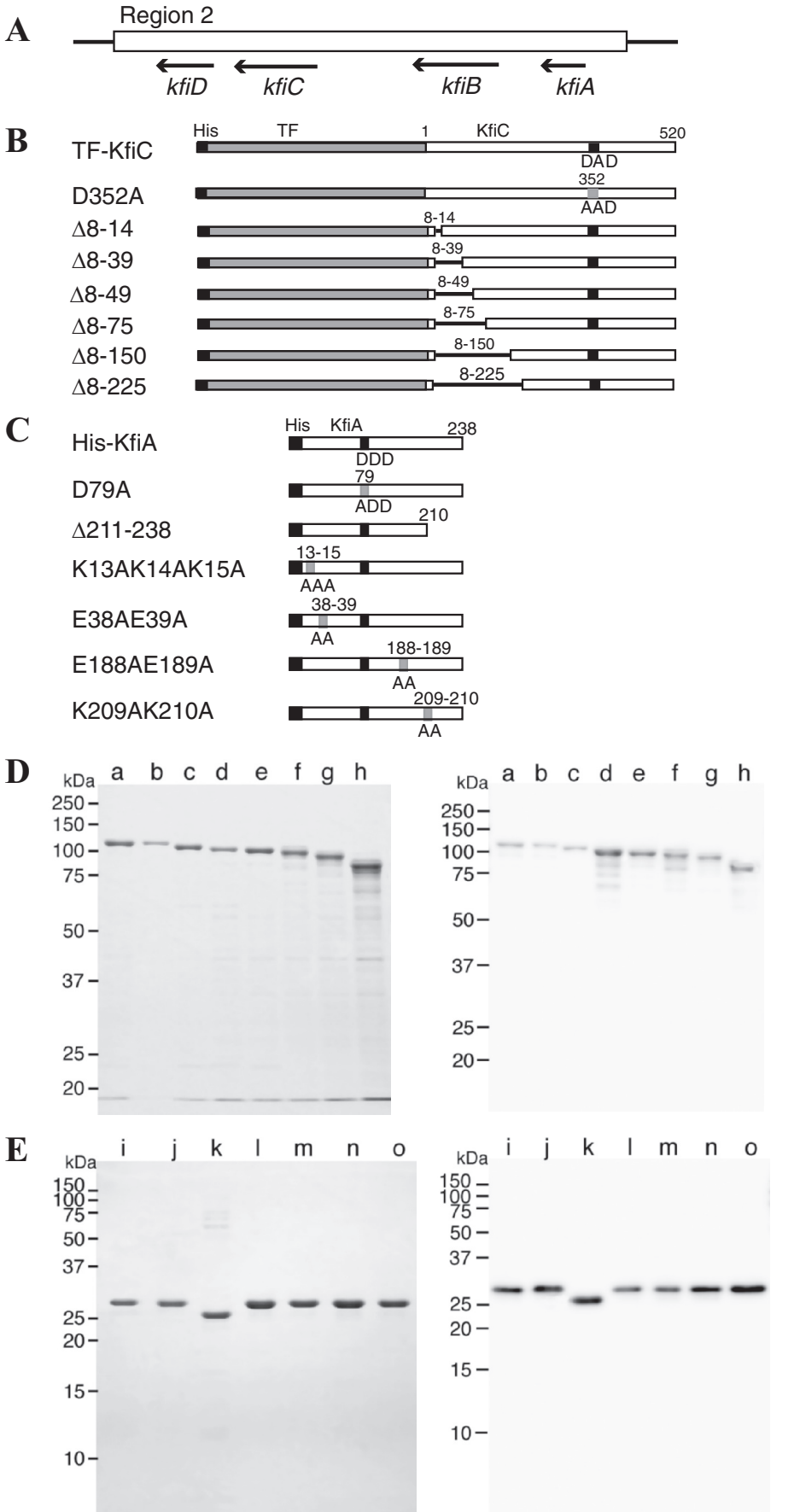
Interaction of KfiC and KfiA

A gene cluster of K5 antigen consists of three functional regions termed 1, 2, and 3. Region 2, located in the middle, is serotype-specific, and it encodes four proteins, KfiA, KfiB, KfiC, and KfiD (Fig. 1*A*), required for synthesis of the K5 polysaccharide (10). KfiD is a UDP-glucose dehydrogenase that catalyzes the formation of UDP-GlcUA from UDP-glucose (11), and KfiB is thought to be a binding protein. In the previous study using crude cell lysate and mutagenesis assays (12, 13), KfiA and KfiC were suggested to possess GlcNAc-T and GlcA-T activity, respectively. Another study showed that recombinant KfiA protein exhibits GlcNAc-T activity (14). However, the enzyme activity of purified KfiC has not been determined yet.

In this study, we expressed and purified both KfiA and KfiC recombinant proteins at a large scale using an *E. coli* expression system, and we characterized them and found that KfiA interacts with KfiC and activates the GlcA-T activity of KfiC. The complex of KfiA and KfiC has GlcNAc-T and GlcA-T activity and exhibits polymerase activity to synthesize the HPR polysaccharide. Our results provide mechanisms of K5 antigen biosynthesis and lead to bioengineering of HS chains.

EXPERIMENTAL PROCEDURES

Materials—K5 polysaccharide (HPR) was purified from the capsule of *E. coli* strain K5 (serotype O10: K5(L):H4, from American Type Culture Collection) following a previous report (8). Heparitinase I (heparan-sulfate lyase from *Flavobacterium heparinum*, EC 4.2.2.8) was purchased from Seikagaku Corp. (Tokyo, Japan); mercury (II) acetate was from Wako Chemical Industries (Osaka, Japan), and UDP-[^{14}C]GlcUA (0.1 Ci/mmol) and UDP-[^{3}H]GalNAc (1.0 Ci/mmol) were from PerkinElmer Life Sciences. UDP-GlcUA, UDP-GalNAc, testicular hyaluronidase (EC 3.2.1.35, type V from sheep testes), and β-glucuronidase (EC 3.2.1.31, type B-10 from bovine liver) were pur-



chased from Sigma. Anti-His tag antibody 27E8 was from Cell Signaling (Boston). Nickel-nitrilotriacetic acid-agarose was from Qiagen (Germantown, MD). Superdex Peptide HR10/30 column, Superdex 30 HR16/60 column, and ECL detection system were purchased from GE Healthcare. Micro BCA protein assay kit was from Pierce. C-terminal peptide fragments of KfiA, KKWPLDIIKETQAIAGYSKLNLELV and LDIIKETQAIAGYSKLNLELVYNVG, were synthesized by Medical and Biological Laboratories (Nagoya, Japan).

Expression and Purification of K5 Proteins—The *kfiA* and *kfiC* genes were amplified from *E. coli* strain K5 genomic DNA using two primers with the corresponding oligonucleotide sequence (*kfiA,* 5′-GGAATTCCATATGATGATTGTTGCAAATATGTCAT-3′, and 5′-CGCGGATCCTTACCCTTCCACATTATACAC-3′, where the cleavage sites of NdeI or BamHI are underlined; *kfiC*, 5′-CATGCCATGGGTATGAACGCAGAATATATAAATTT-3′, and 5′-CCGCTCGAGTTGTTCAATTATTCCTGATACATC-3′, where the cleavage sites of NcoI or XhoI are underlined).

The *kfiA* gene was cloned into the pET15b$^+$ expression vector (Novagen) to yield the N-terminal His_6-tagged protein. The resulting expression plasmids (pETHisKfiA) were transformed into *E. coli* BL21(DE3)pGro7 cells (Novagen). The transformants were cultured in 2× YT medium containing 50 μg/ml ampicillin and 20 μg/ml chloramphenicol for 3 h at 37 °C. The culture was cooled to 22 °C and then supplemented with isopropyl β-D-thiogalactopyranoside (1 mM) and arabinose (1 mg/ml) for induction of KfiA-tagged protein and chaperone, respectively. After 14 h of culture, the cells were harvested by centrifugation and lysed by sonication in 50 mM Tris-HCl, pH 7.4, and 0.5 M NaCl (buffer A).

The *kfiC* gene was cloned into the pENTR11 (Invitrogen), and the resulting plasmid was named pENTR-KfiC. To construct the *E. coli* expression plasmid pColdDEST, the Gateway® DEST cassette was amplified from pDEST38 (Invitrogen) by PCR with the primers 5′-CCGATGGTGATGACCTGACCGTCGGG-3′ and 5′-ACAAGTTTGTACAAAAAAGCTG-3′ and digested with XbaI. The resulting fragment was introduced into a Ecl36II-XbaI site of pColdII (Takara Bio). The *E. coli* trigger factor (TF) sequence was amplified from DH10B cells (Invitrogen) by PCR with the primers 5′-GGAATTCCATATGCAAGTTTCAGTTGAAAC-3′ and 5′-GGAATTCCATATGCGCCTGCTGGTTCATCA-3′, followed by NdeI digestion, and cloned into an NdeI site of pColdDEST. The resulting plasmid was named pColdTFDEST. Then the construct pColdTFDEST was subjected to the Gateway® LR reaction with entry vector, pENTR-KfiC, using LR Clonase™ enzyme mixture (Invitrogen) according to the protocols recommended in the manufacturer's manual. The resulting expression plasmid named pColdTFKfiCDEST was transformed into *E. coli* BL21(DE3) Codonplus RIL cells (Stratagene) to yield the N-terminal His-tagged TF fusion protein. The transformants were cultured in LB medium containing 100 μg/ml ampicillin for 8 h at 37 °C. The culture was cooled to 15 °C, and then supplemented with isopropyl β-D-thiogalactopyranoside (0.5 mM) for induction of KfiC fusion protein and chaperone, prospectively. After 14 h of culture, the cells were harvested by centrifugation and lysed by sonication in buffer A.

The cell lysate containing His-tagged protein was applied to nickel-nitrilotriacetic acid-agarose gel, and His-tagged protein was eluted with buffer A containing 250 mM imidazole. The eluted proteins were dialyzed against 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 2 mM 2-mercaptoethanol.

Preparation of Truncated and Point Mutants of KfiA and KfiC—DNA constructs with truncated and point mutants were prepared using PrimeSTAR® mutagenesis basal kit (Takara) with the plasmids containing *kfiA* and *kfiC* genes as the templates, according to the manufacturer's instructions. DNA fragments encoding ^{79}ADD of KfiA and ^{352}AAD of KfiC were constructed with the expression vectors to yield His-KfiA D79A and TF-KfiC D352A, using pairs of primers that were 5′-CTTACAGCTGATGATATTATTTACCCT-3′, 5′-ATCATCAGCTGTAAGTACGATCATATC-3′ and 5′-CCTTTCAAGCTGCAGATGATCTTTCTCATC-3′, 5′-GAAAGATCATCTGCAGCTTGAAAGGTGATG-3′, respectively. The pair of primers used to delete the C-terminal 28-amino acid residues of KfiA and to yield His-KfiA Δ211–259 was 5′-GGCAGCCATATGATGATTGTTGCAAATATGTCAT-3′ and 5′-CGGGTACCTTATTTTTTTGTAAATGTGTTCCAAAG-3′. The pairs of primers used to mutate the ^{13}KKE^{15}, ^{38}EE^{39}, ^{188}EK^{189}, and ^{209}KK^{210} amino acid residues of KfiA to AAA, AA, AA, and AA and to yield His-KfiA K13A/K14A/E15A, His-KfiA E38A/E39A, His-KfiA E188A/K189A, and His-KfiA K209A/K210A were as follows: 5′-CCTCGAGCAGCAGCGTTGGTGCATTCTATACAAAG-3′ and 5′-ACCAACGCTGCTGCTCGAGGTGGGTATGATGACAT-3′; 5′-AGTTTGCAGCAATTCCTGAGGAATTAGATGGTT-3′ and 5′-GGAATTGCTGCAAACTCATTCAGGCAAAGATTA-3′; 5′-CCAGAGCAGCAAACTGGCTAAGAGAGGTCTCAT-3′ and 5′-CAGTTTGCTGCTCTGGGAACACATATCATACCA-3′; 5′-TTTACAGCAGCATGGCCTTTAGACATCATAAAAGA-3′ and 5′-AGGCCATGCTGCTGTAAATGTGTTCCAAAGTCCTT-3′, respectively. The pairs of primers used to delete the N-terminal amino acid residues of KfiC at 8–14, 8–39, 8–49, 8–75, 8–150, and 8–225 and to yield TF-KfiC Δ8–14, TF-KfiC Δ8–39, TF-KfiC Δ8–49, TF-KfiC Δ8–75, TF-KfiC Δ8–150, and TF-KfiC Δ8–225 were as follows: 5′-

FIGURE 1. **Capsular gene cluster of *E. coli* K5 and the recombinant K5 proteins expressed.** *A,* region 2 of a capsular gene cluster of *E. coli* strain K5 contains four genes (*kfiA*, *kfiB*, *kfiC*, and *kfiD*). *B,* constructs of the recombinant KfiC and the truncated and point mutants having N-terminal His tag and TF fusion protein. *C,* constructs of the recombinant KfiA and the truncated and point mutants having N-terminal His tag. *D,* SDS-PAGE at 10% polyacrylamide gel followed by Coomassie Brilliant Blue staining (*left*) and Western blotting immunostaining with anti-His antibody (*right*) of affinity-purified KfiC and the mutant proteins (*lane a,* TF-KfiC; *lane b,* TF-KfiC D352A; *lane c,* TF-KfiC Δ8–14; *lane d,* TF-KfiC Δ8–39; *lane* e, TF-KfiC Δ8–49; *lane f,* TF-KfiC Δ8–75; *lane g,* TF-KfiC Δ8–150, and *lane h,* TF-KfiC Δ8–225). *E,* SDS-PAGE at 15% polyacrylamide gel followed by Coomassie Brilliant Blue staining (*left*) and Western blotting immunostaining with anti-His antibody (*right*) of affinity-purified KfiA and the mutant proteins (*lane i,* His-KfiA; *lane j,* His-KfiA D79A; *lane k,* His-KfiA Δ211–238; *lane l,* His-KfiA K13AK14AK15A; *lane m,* His-KfiA E38AE39A; *lane n,* His-KfiA E188AE189A, and *lane o,* His-KfiA K209AK210A). Molecular size standards are indicated on the *left side* of the panels.

TATATAAATTTAGGGACAAATATTGGTGCT-3′ and 5′-TCCCTAAATTTATATATTCTGCGTTCAT-3′; 5′-TATATAAATTCGCCTTTAAAAGGTAACGAT-3′ and 5′-AAGGCGAATTTATATATTCTGCGTTCAT-3′; 5′-TATATAAATCACAAAAGAATAAACGAATAC-3′ and 5′-TTTTGTGATTTATATATTCTGCGTTCAT-3′; 5′-TATAAATTCTTATTTATTGGGATATGCA-3′ and 5′-AATAAGAATTTATATATTCTGCGTTCAT-3′; 5′-TATAAATCCCAAACAAAAAGATTTTTAT-3′ and 5′-GTTTGGGATTTATATATTCTGCGTTCAT-3′; 5′-TATAAATCAATTTAACAAATGTCTACGA-3′ and 5′-TAAATTGATTTATATATTCTGCGTTCAT-3′, respectively. The mutations were confirmed by sequencing with the Big Dye terminator cycle sequencing kit (Applied Biosystems).

Preparation of HPR Oligosaccharides—HPR oligosaccharides were prepared as described previously (16), with a slight modification. Briefly, HPR polysaccharide (10 mg) was digested with heparitinase I (10 milliunits) in 0.1 M sodium acetate, pH 7, containing 5 mM calcium acetate at 30 °C for 16 h, and the digest was heated in boiling water for 5 min. After centrifugation, the even-numbered oligosaccharides containing unsaturated GlcUA at the nonreducing termini were desalted with a Superdex 30 HiLoad 16/60 column and then treated with 70 mM mercury(II) acetate solution, pH 5.0, at room temperature for 10 min to remove the unsaturated GlcUA residue. The odd-numbered oligosaccharides containing GlcNAc residue at the nonreducing termini were directly passed through a column (10-ml bed volume) of Dowex 50W-X8 (H^+ form) to remove mercury ions and then separated by gel filtration chromatography with a Superdex 30 HiLoad 16/60 column. Some fractions contained HPR heptasaccharide (HPR-7). The structures of the oligosaccharides were determined by MALDI-TOF mass spectrometry and electrospray ionization-mass spectrometry analyses (17). The amount of HPR oligo- and polysaccharides was determined by carbazole assay method with GlcUA monosaccharide as standard (18).

Treatment of Nonreducing Terminus of HPR Polysaccharide—HPR polysaccharide (7 mg) was treated by β-glucosidase (5.0 units) in 0.1 M NaOAc, pH 5.2, containing 0.15 M NaCl (500 μl) at 37 °C for 18 h. The reaction mixture was heated in boiling water for 3 min and chilled to room temperature. The polysaccharide-containing GlcNAc residue at the nonreducing terminus (GlcNAc-HPR, 5.3 mg) was separated by gel filtration chromatography on a Superdex 6 HR10/30 column.

Measurement of Enzyme Activities Using Radioisotope Donor Substrates—GlcNAc-T, GlcA-T, and HPR polymerase activities of the recombinant enzymes were measured using radioisotope donor substrates. For the standard assay of GlcNAc-T, a 50-μl mixture containing 50 mM BisTris-HCl, pH 6.5, 2 mM $MnCl_2$, UDP-[^{3}H]GlcNAc (0.1 μCi, 300 pmol) as the donor substrate, and HPR polysaccharide (300 pmol) as the acceptor substrate was incubated with the recombinant enzyme (60 pmol) at 30 °C for 60 min. The final concentration of NaCl to be responsible for the enzyme solution was ~5 mM. For the standard assay of GlcA-T, a reaction mixture of UDP-[^{14}C]GlcUA (0.1 μCi, 300 pmol) and GlcNAc-HPR (300 pmol) in 50 mM BisTris-HCl, pH 6.5, 2 mM $MnCl_2$ was incubated with the recombinant enzymes (either 60 pmol) at 30 °C for 60 min. The final concentration of NaCl to be responsible for the enzyme solutions was ~10 mM. For the standard assay of the polymerase, a reaction mixture of HPR-7 (100 pmol) as the acceptor substrate and a pair of UDP-[^{14}C]GlcUA (0.1 μCi, 3.3 nmol) and UDP-GlcNAc (3.3 nmol) or that of UDP-[^{3}H]GlcNAc (0.1 μC, 3.3 nmol) and UDP-GlcUA (3.3 nmol) as the donor substrates in 50 mM BisTris-HCl, pH 6.5, 2 mM $MnCl_2$ were incubated with the recombinant enzymes (either at 60 pmol) at 30 °C for 18 h. The final concentration of NaCl to be responsible for the enzyme solutions was ~10 mM. After each reaction mixture was heated in boiling water for 1 min, the radiolabeled product was separated by a Superdex Peptide HR10/30 column and measured by liquid scintillation counting. The enzyme activities were determined by calculating the amount of the incorporated radioactive sugars.

SDS-PAGE and Western Blotting—SDS-PAGE was carried out on a 10 and 15% polyacrylamide gel by the method of Laemmli (19). Proteins were detected by Coomassie Brilliant Blue staining. For Western blotting, proteins on the SDS-polyacrylamide gel were transferred onto a polyvinylidene difluoride transfer membrane (Immobilon-P, Millipore). The membrane was treated with 50 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 0.05% Tween 20 (TBS-T) containing 5% skim milk for 1 h and then with an anti-His antibody. After washing with TBS-T, the membrane was treated with a horseradish peroxidase-conjugated secondary antibody, and the signal was detected using the enhanced chemiluminescence detection system (Western Lightning Plus, PerkinElmer Life Sciences) and a lumino-image analyzer (LAS-4000 mini, Fujifilm). Protein content was determined using a micro BCA protein assay kit (Pierce) with bovine serum albumin as standard.

RESULTS

Expression and Purification of K5 Proteins—Initially, we obtained both *kfiA* and *kfiC* genes by PCR from *E. coli* strain K5 genomic DNA (Fig. 1*A*), cloned into expression vectors, and expressed and purified tag-attached recombinant enzymes. Similarly, we prepared various mutants (Fig. 1, *B* and *C*) as described under "Experimental Procedures." Trigger factor (TF) is a ribosome-associated molecule in *E. coli* and acts as a chaperon to promote a new synthesized protein folding. Using TF as a fusion protein of KfiC, we successfully and efficiently expressed the recombinant protein as a soluble protein without inclusion body formation. The purified intact TF-KfiC and TF-KfiC D352A mutants predominantly migrated as a major band of ~112 kDa, and the truncated KfiC mutants TF-KfiC Δ8–14, TF-KfiC Δ8–39, TF-KfiC Δ8–49, TF-KfiC Δ8–75, TF-KfiC Δ8–150, and TF-KfiC Δ8–225 did at ~111, 108, 107, 104, 95, and 86 kDa, respectively, on a 10% SDS-polyacrylamide gel and Western blotting membrane (Fig. 1*D*). The purified His-KfiA, His-KfiA D79A, His-KfiA K13A/K14A/K15A, His-KfiA E38A/E39A, His-KfiA E188A/E189A, and His-KfiA K209A/K210A point mutants migrated as a major band at ~30 kDa, respectively, and His-KfiA Δ211–238 truncated mutant did at ~26 kDa on a 15% SDS-polyacrylamide gel and Western blotting membrane (Fig. 1*E*). The apparent molecular mass of the expressed proteins corresponded well to their calculated molecular mass of TF-KfiC, TF-KfiC D352A, TF-KfiC Δ8–14,

TABLE 1
Enzyme activities of KfiA and KfiC were measured by radiolabeled sugar nucleotide donors with various HPR acceptors

No.	Enzyme(s)	Acceptor	Donor(s)	Type of activity	Reaction time	Activity	%[a]
					h	*pmol/min/pmol enzyme*	
1.	KfiA	HPR	UDP-[^{3}H]GlcNAc	GlcNAc-T	1	7.54 ± 0.37[b]	89.7
2.	KfiC	HPR	UDP-[^{14}C]GlcUA	GlcA-T	1	<0.05	<0.6
3.	KfiC + KfiA	HPR	UDP-[^{14}C]GlcUA	GlcA-T	1	1.46 ± 0.24	17.4
4.	KfiC + KfiA	HPR	UDP-[^{14}C]GlcUA + UDP-GlcNAc	Polymerase	18	7.85 ± 1.33	93.3
5.	KfiA	GlcNAc-HPR	UDP-[^{3}H]GlcNAc	GlcNAc-T	1	<0.05	<0.6
6.	KfiC	GlcNAc-HPR	UDP-[^{14}C]GlcUA	GlcA-T	1	<0.05	<0.6
7.	KfiC + KfiA	GlcNAc-HPR	UDP-[^{14}C]GlcUA	GlcA-T	1	8.41 ± 0.50	100
8.	KfiC + KfiA	GlcNAc-HPR	UDP-[^{3}H]GlcNAc + UDP-GlcA	Polymerase	18	7.70 ± 0.56	91.6
9.	KfiC + KfiA	HPR-7	UDP-[^{3}H]GlcNAc + UDP-GlcUA	Polymerase	18	7.01 ± 1.14	83.4
10.	KfiC + KfiA	HPR-7	UDP-[^{14}C]GlcUA + UDP-GlcNAc	Polymerase	18	6.86 ± 0.98	81.6

[a] The relative activity when GlcA-T activity using KfiC and KfiA mixed enzyme, GlcNAc-HPR as acceptor, and UDP-[^{14}C]GlcUA as donor is 100%.
[b] Data are the mean values of three independent experiments ± standard deviations.

TF-KfiC Δ8–39, TF-KfiC Δ8–49, TF-KfiC Δ8–75, TF-KfiC Δ8–150, and TF-KfiC Δ8–225 (112,076, 112,032, 111,194, 108,408, 107,359, 104,367, 95,368, and 86,578 Da, respectively) and His-KfiA, His-KfiA D79A, His-KfiA Δ211–238, His-KfiA K13A/K14A/K5A, His-Kfia E38A/E39A, His-KfiA E188A/E189A, and His-KfiA K209A/K210A point mutants (29,626, 29,582, 26,499, 29,453, 29,510, 29,511, and 29,511 Da, respectively).

Preparation of HPR Oligosaccharides and GlcNAc-HPR Polysaccharide—As defined substrates were necessary for determination of GlcA-T and GlcNAc-T activities, we prepared oligosaccharides and HPR polysaccharides with specific nonreducing termini as follows. The odd-numbered oligosaccharides (*e.g.* heptasaccharide), whose nonreducing terminus was the GlcNAc residue, were obtained by mercury acetate treatment of even-numbered oligosaccharides (*e.g.* octasaccharide) containing unsaturated GlcUA residue at the nonreducing termini, which were digested with heparitinase I. The structure of the heptasaccharide (HPR-7) was confirmed by MALDI-TOF mass spectrometry analysis (data not shown). GlcNAc HPR polysaccharide, whose nonreducing terminus was GlcNAc residue, was obtained by treatment of HPR polysaccharide with β-glucuronidase to remove GlcUA residue at the nonreducing terminus.

Measurements of Enzyme Activities of KfiA and KfiC—Table 1 shows the summary of enzyme activities of the recombinant K5 proteins. His-KfiA exhibited GlcNAc-T activity, using HPR polysaccharide as acceptor and UDP-[^{3}H]GlcNAc as donor, consistent with the previous report (14). In contrast, TF-KfiC showed no GlcA-T activity, using HPR polysaccharide acceptor and UDP-[^{14}C]GlcUA donor. His-KfiA exhibited no GlcA-T activity, and TF-KfiC had no GlcNAc-T activity (data not shown). Interestingly, a mixture of TF-KfiC and His-KfiA showed certain levels of [^{14}C]GlcUA incorporation into HPR polysaccharide, which were elevated when reacted for 18 h with both UDP-[^{14}C]GlcUA and UDP-GlcNAc as donors, suggesting chain polymerization.

Next, we examined the GlcA-T activity of the enzyme mixture, using GlcNAc-HPR prepared with glucuronidase as acceptor substrate. His-KfiA did not transfer GlcNAc to the acceptor substrate, confirming the absence of GlcUA at the nonreducing terminus of HPR. TF-KfiC alone did not show GlcA-T activity, even using GlcNAc-HPR as acceptor substrate. In contrast, the mixture of TF-KfiC and His-KfiA transferred [^{14}C]GlcUA onto GlcNAc-HPR, exhibiting GlcA-T activity. The activity (8.41 ± 0.50 pmol of GlcUA/min/pmol of each enzyme) was comparable or even higher than that of GlcNAc-T of KfiA (7.54 ± 0.37 pmol of GlcNAc/min/pmol of enzyme). However, when native HPR polymer was used as acceptor, the enzyme mixture showed lower GlcA-T activity (1.46 ± 0.24 pmol of GlcUA/min/pmol of enzyme), suggesting that biosynthesis of HPR polysaccharide by *E. coli* strain K5 is prone to terminate when GlcUA residue was transferred at the nonreducing end.

When both UDP-[^{3}H]GlcNAc and UDP-GlcUA were used as donor substrates, the enzyme mixture certainly incorporated [^{3}H]GlcNAc into GlcNAc-HPR (7.70 ± 0.56 pmol of GlcNAc/min/pmol of enzyme). When UDP-[^{14}C]GlcUA and UDP-GlcNAc were used as donors, the enzyme mixture also incorporated [^{14}C]GlcUA into HPR polymer (7.85 ± 1.33 pmol of GlcUA/min/pmol of enzyme). These results indicate that the mixture of KfiA and KfiC catalyzes HPR polymerization by alternately transferring GlcNAc and GlcUA residues.

Assay Conditions for GlcNAc-T and GlcA-T Activities—We measured GlcA-T activity of the enzyme mixture at different ratios, adjusting the final NaCl concentration to 50 mM (Fig. 2*A*). GlcA-T activity increased as the ratio of KfiA/KfiC increased. Addition of KfiA at a 2-fold molar ratio to that of KfiC showed ~1.5-fold GlcA-T activity compared with an equimolar ratio of KfiA and KfiC. The activity of a 5-fold addition of KfiA was similar to that of the 2-fold addition. KfiA and KfiC alone showed no GlcA-T activity. Addition of KfiC had no effect on the GlcNAc-T activity of KfiA (data not shown).

We then examined the effects of NaCl concentrations on GlcNAc-T and GlcA-T activities of His-KfiA and the mixture of TF-KfiC and His-KfiA (Fig. 2*B*). A maximal activity of both transferases was obtained in the reaction buffer containing 50 mM BisTris-HCl, pH 6.5, 2 mM $MnCl_2$ and the lowest concentration of NaCl (5–10 mM). Although GlcNAc-T activity did not change at different NaCl concentrations up to 205 mM, GlcA-T activity was significantly attenuated by increasing NaCl concentrations. The GlcA-T activity was ~50 and ~25% at 60 and 160 mM NaCl, respectively, compared with that under the lowest NaCl concentration (10 mM). Addition of KCl in place of NaCl showed similar effects (data not shown).

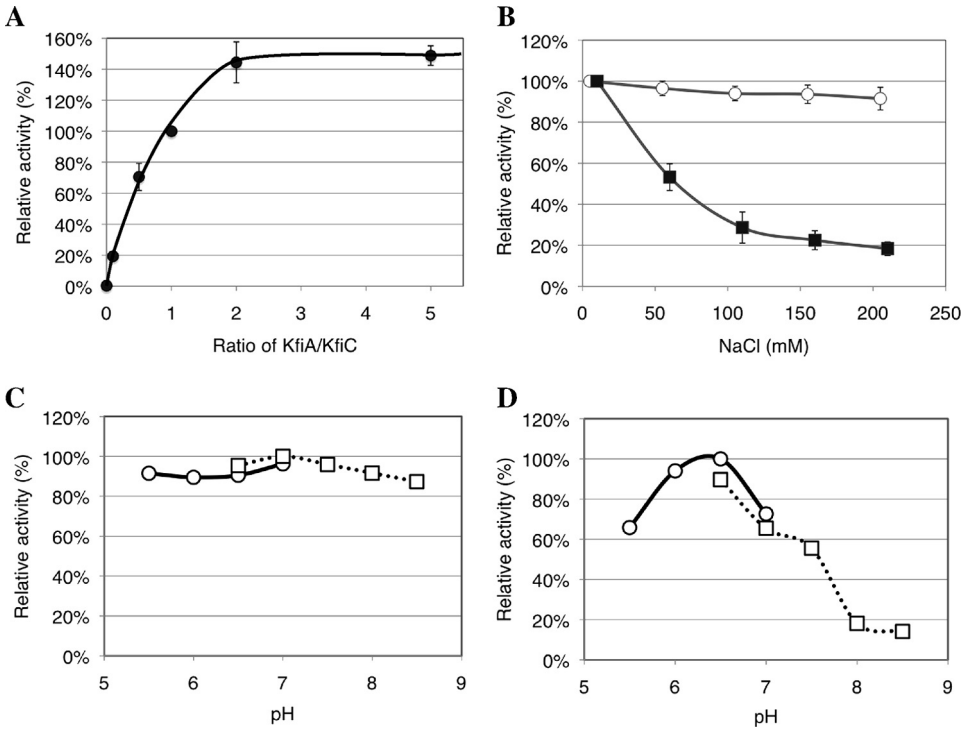


FIGURE 2. **Effects on GlcNAc-T and GlcA-T activities of KfiA and KfiC.** *A,* GlcA-T activity was measured at different ratios of KfiC and KfiA. A reaction mixture (50 μl) containing TF-KfiC (60 pmol) and His-KfiA (0 ~ 300 pmol), of which the final NaCl concentration was adjusted to 50 mM, was incubated at 30 °C for 60 min. The activity is shown as a percent of the [^{14}C]GlcUA radioactivity incorporated into GlcNAc-HPR and that of an equimolar mixture (either 60 pmol) of TF-KfiC and His-KfiA (100%). *B,* GlcNAc-T (*open circles*) and GlcA-T activities (*closed squares*) of KfiA and equimolar mixture of KfiC and KfiA were measured at different NaCl concentrations. The reaction mixtures containing 5–200 mM NaCl were incubated at 30 °C for 60 min with the enzymes, acceptor, and donor substrates. The data in the table are relative percentages of the radioactivity incorporated in comparison with the assay at the lowest NaCl concentration (100%). *C,* GlcNAc-T. *D,* GlcA-T activities were measured under standard assay conditions with different pH values of buffer conditions. The buffers used were BisTris-HCl (pH 5.5–7.0, *open circles*) and Tris-HCl (pH 6.5– 8.5, *open squares*). The data are shown as a percent of the radioactivity incorporated and that of the reaction containing Tris-HCl buffer at pH 7.0 on GlcNAc-T and BisTris-HCl buffer at pH 6.5 on GlcA-T (100%), respectively.

TABLE 2

Effects of divalent cations and chelate reagent on the GlcUA transferase activity of KfiA and HPR polymerase activity of KfiC and KfiA mixture

The enzyme activities were measured as described under "Experimental Procedures." The reaction mixture (50 μl) containing 2 mM divalent metal ion (Mn^{2+}, Mg^{2+}, Ca^{2+}, Ba^{2+}, Cd^{2+}, Co^{2+}, Cu^{2+}, or Fe^{2+}) or 20 mM EDTA substituted for the metal ion was incubated at 30 °C for 60 min with the enzymes, acceptor, and donor substrates. The data in the table are the relative percentages of the radioactivity incorporated in comparison with the assay containing Mn^{2+} ion (100%).

Metal ion or reagent	Relative activity	
	GlcNAc-T	GlcA-T
	%	%
Mn^{2+}	100.0	100.0
Mg^{2+}	10.4	19.4
Ca^{2+}	3.0	1.7
Ba^{2+}	2.2	2.5
Cd^{2+}	2.5	2.0
Co^{2+}	56.3	3.2
Cu^{2+}	0.2	2.1
Fe^{2+}	19.4	13.7
EDTA	1.9	1.6

The GlcNAc-T (Fig. 2*C*) and GlcA-T (Fig. 2*D*) activities were optimal at pH 7.0 and 6.5, respectively. pH conditions examined in a range of pH 5.5– 8.5 affected GlcNAc-T activity little, but it did affect GlcA-T activity. The relative activity of GlcNAc-T was 90% optimal at pH 6.5 for GlcA-T in BisTris-HCl buffer, whereas that of GlcA-T was ~66% optimal at pH 7.0 for GlcNAc-T in Tris-HCl buffer. The relative activity of GlcNAc-T and GlcA-T was 90 and 66% at pH 5.5 in BisTris-HCl buffer and 88 and 14% at pH 8.5 in Tris-HCl buffer, respectively, compared with that under the optimal conditions. Thus, all the following enzymatic reactions were carried out in 50 mM BisTris-HCl buffer, pH 6.5.

Next, we examined the effects of divalent metal ions on the GlcNAc-T and GlcA-T activities of His-KfiA and the mixture of TF-KfiC and His-KfiA (Table 2). Maximal activity was obtained in the presence of Mn^{2+} ion (2 mM) for both transferase activities. The presence of Co^{2+} ion showed 56 and 3% that of GlcNAc-T and GlcA-T activity. Mg^{2+} and Fe^{2+} showed 10–20% that of both activities, as compared with that of Mn^{2+} ion. Ca^{2+}. Ba^{2+}, Cd^{2+}, Cu^{2+}, and EDTA showed little activity (0 ~ 3%). We then examined the effects of Mn^{2+} concentrations on GlcNAc-T and GlcA-T activities of His-KfiA and the mixture of TF-KfiC and His-KfiA (Table 3). A maximal activity was obtained at 2 mM $MnCl_2$ in a 60-min reaction for both GlcNAc-T and GlcA-T. Both activities were attenuated by decreasing $MnCl_2$ concentrations. Although GlcA-T activity at 20 mM $MnCl_2$ decreased to ~67% that at 2 mM $MnCl_2$, GlcNAc-T activity was affected only a little.

Enzyme Activities of the Point Mutants of KfiA and KfiC—The fact that the enzyme mixture of KfiA and KfiC indicated GlcA-T activity implies that KfiC exhibits GlcA-T activity in the presence of KfiA or that KfiA acquires GlcA-T activity in the presence of KfiC. To determine which enzyme transfers GlcUA, we measured activity using the enzymes with point mutations.

The D*X*D motif that binds the UDP-sugar donor substrate with Mn^{2+} ion is essential for glycosyltransferase activity. Both KfiA and KfiC have a D*X*D motif in the glycosyltransferase active site. We constructed point mutants replacing an aspartic acid residue with an alanine residue at position ^{79}DDD of KfiA and ^{352}DAD of KfiC to ^{79}ADD and ^{352}AAD, respectively. The recombinant proteins, His-KfiA D79A and TF-KfiC D352A, were purified by nickel affinity chromatography and dialyzed.

His-KfiA D79A point mutant showed no GlcNAc-T activity (Table 4 and Fig. 3*A*). The mixture of intact TF-KfiC and His-KfiA D79A represented the GlcA-T activity comparable with a mixture of intact enzymes. In contrast, TF-KfiC D352A mutant showed no GlcA-T activity with intact or mutant His-KfiA.

TABLE 3

GlcNAc and GlcUA transferase activity of KfiA and equimolar mixture of KfiC and KfiA at various concentrations of Mn^{2+} ions

The enzyme activities were measured as described under "Experimental Procedures." The reaction mixtures containing 0–20 mM $MnCl_2$ was incubated at 30 °C for 60 min. The data in the table are the relative percentages of the radioactivity incorporated in comparison with the assay containing 2.0 mM $MnCl_2$ (100%).

$MnCl_2$	Relative activity	
	GlcNAc-T	GlcA-T
mM	%	%
0.02	33.2	26.7
0.2	77.0	60.5
2.0	100.0	100.0
20.0	99.1	67.1

TABLE 4

Enzyme activities of the recombinant proteins of intact KfiA, KfiC, and their point mutants

GlcNAc transferase activity was measured using HPR polymer acceptor and UDP-[^{3}H]GlcNAc donor with intact KfiA (wild type (WT)) or D79A point mutant. GlcUA transferase activity was measured using GlcNAc-HPR acceptor and two donors of UDP-[^{14}C]GlcUA and UDP-GlcNAc with equimolar mixture of intact KfiC (WT) or D362A mutant and intact KfiA (WT) or D79A mutant. The polymerase activity was measured using HPR-7 acceptor and two donors of UDP-[^{3}H]GlcNAc and UDP-GlcUA with equimolar mixture of intact KfiC (WT) or D362A mutant and intact KfiA (WT) or D79A mutant. The data in the table represent the average of three independent experiments and the standard deviations.

Type of activity	Enzymes		Activity
	KfiC	KfiA	
			pmol/min/pmol enzyme
GlcNAc-T		WT	7.54 ± 0.37
		D79A	<0.05
GlcA-T	WT	WT	8.41 ± 0.50
	WT	D79A	8.70 ± 0.67
	D362A	WT	<0.05
	D362A	D79A	<0.05
Polymerase	WT	WT	7.01 ± 1.14
	WT	D79A	<0.05
	D362A	WT	<0.05
	D362A	D79A	<0.05

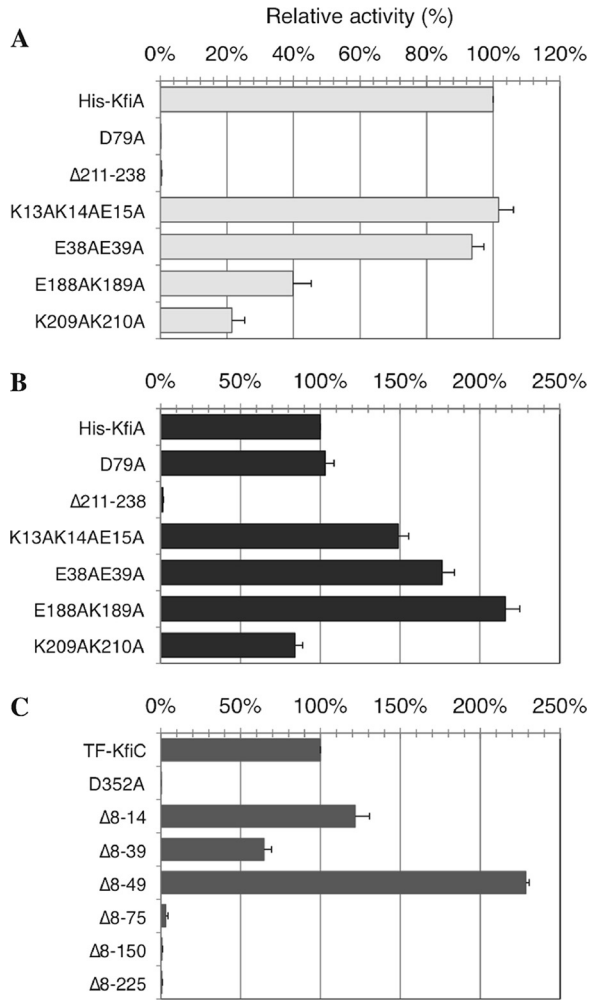


FIGURE 3. **Enzyme activities of the truncated and point mutants of KfiA and KfiC.** *A*, GlcNAc-T activity of the KfiA mutants was measured as described under "Experimental Procedures." The data are shown as a percent of the [^{3}H]GlcNAc radioactivity incorporation to HPR and that of the reaction containing the wild type His-KfiA (100%). *B*, GlcA-T activity of the wild type TF-KfiC with the mutants of KfiA was measured. The data are shown as a percent of the [^{14}C]GlcUA radioactivity incorporation to GlcNAc-HPR and that of the reaction containing wild type of His-KfiA and TF-KfiC (100%). *C*, GlcA-T activity of the mutants of TF-KfiC with the wild type His-KfiA was measured. The data are shown as a percent of the radioactivity incorporation to GlcNAc-HPR and that of the reaction containing wild type of TF-KfiC and His-KfiA (100%).

These results clearly demonstrate that KfiC has the GlcA-T activity in the enzyme mixture.

A mixture of either mutant or both mutants at the D*X*D motifs of KfiA and KfiC did not show polymerase activity when HPR-7 acceptor, radiolabeled UDP-GlcNAc, and nonlabeled UDP-GlcNAc were used as donor substrates. This indicates that the polymerization occurs by alternate transfer of GlcNAc by KfiA and GlcUA by KfiC.

Then we obtained various point mutants of KfiA replaced ionic amino acid cluster (for example lysine and glutamic acid) at positions ^{13}KKE^{15}, ^{38}EE^{39}, ^{188}EK^{189}, and ^{209}KK^{210} with non-ionic amino acid alanine to yield His-KfiA K13A/K14A/E15A, His-KfiA E38A/E39A, His-KfiA E188A/K189A, and His-KfiA K209A/K210A. His-KfiA K13A/K14A/E15A and His-KfiA E38A/E39A showed similar activity of GlcNAc-T to intact His-KfiA, and ~1.5–1.7 times higher activation activity of GlcA-T than intact His-KfiA (Fig. 3*B*). His-KfiA E188A/E189A had lower activity (~40%) of GlcNAc-T but 2.2 times activation activity of GlcA-T than intact KfiA. His-KfiA K209A/K210A had further lower activity (~20%) of GlcNAc-T and relatively lower activation activity (~80%) of GlcA-T than intact enzyme.

Enzyme Activities of the Truncated Mutants of KfiA and KfiC—To determine regions responsible for enzymatic activity, we used various truncated mutants. A truncated mutant of KfiA that lacked the C-terminal 28 amino acid residues (His-KfiA Δ211–238) exhibited no GlcNAc-T activity (Fig. 3*A*). The C-terminal truncated mutant of KfiA did not activate GlcA-T activity of KfiC (Fig. 3*B*). The C-terminal sequence may be essential for both activities. Two synthetic peptides consisting of 25 amino acid residues at the C-terminal region of KfiA (KKWPLDIIKETQAIAGYSKLNLELV and LDIIKETQAIAGYSKLNLELVYNVG) were added to the GlcA-T assay system with or without KfiA. The peptides showed neither inhibition nor activation effects on GlcA-T up to 1 mM (data not shown).

N-terminal truncated mutants of KfiC were used to measure GlcA-T activity with intact His-KfiA (Fig. 3*C*). TF-KfiC Δ8–14, TF-KfiC Δ8–39, and TF-KfiC Δ8–49 indicated ~1.2, 0.6, and 2.3 times the GlcA-T activity of intact TF-KfiC, respectively. In comparison, further truncated mutants TF-KfiC Δ8–75 showed a little GlcA-T activity (~3%), and TF-KfiC Δ8–150, and TF-KfiC Δ8–225 had practically no activity of GlcA-T. The

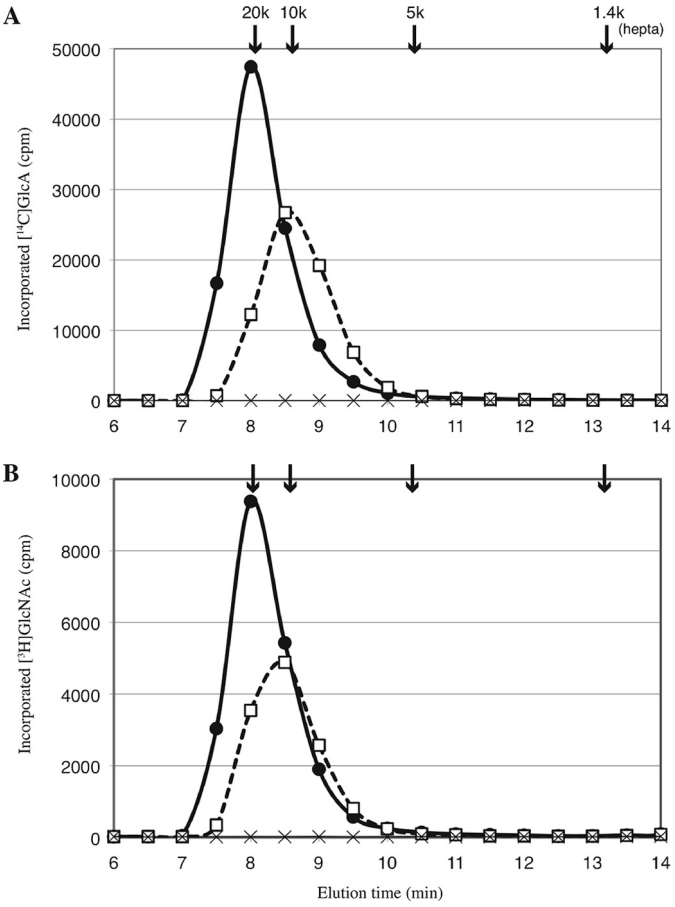


FIGURE 4. **Polymerization reaction catalyzed by KfiC and KfiA.** A mixture of the purified TF-KfiC (60 pmol) and His-KfiA proteins (60 pmol), containing HPR-7 (100 pmol) as acceptor substrate, was incubated with UDP-[^{14}C]GlcUA (0.1 μCi, 3.3 nmol) and UDP-GlcNAc (3.3 nmol) (*A*) or UDP-GlcUA (3.3 nmol) and UDP-[^{3}H]GlcNAc (0.1 μCi, 3.3 nmol) (*B*) at 30 °C for 8 h (*open square*) and 18 h (*solid circle*) as described under "Experimental Procedures." The reactions were applied to a Superdex Peptide HR10/30 column, and the radioactivity in the fractions was measured. A portion of the reaction products was treated with heparitinase I (*crosses*; both 8- and 18-h polymerization reactions). The *arrows* denote the positions of hyaluronan sodium salt standards (*20k*, M_r 20,000; *10k*, M_r 10,000; and *5k*, M_r 5000) and HPR heptasaccharide sodium salt (HPR-7 (*hepta*); M_r 1425).

single reaction of all KfiC mutants that we examined showed no GlcA-T activity without KfiA (data not shown), same as intact KfiC.

Polymerization Reaction of KfiA and KfiC—We further investigated the polymerase activity of the enzyme mixture, using HPR-7 (GlcNAc-(GlcUA-GlcNAc)$_3$) oligosaccharide as an acceptor substrate and UDP-GlcUA and UDP-GlcNAc as donor substrates, either of which was radioisotope-labeled. An equimolar enzyme mixture of TF-KfiC and His-KfiA produced ~10 and 20 kDa of [^{3}H]GlcNAc- or [^{14}C]GlcUA-incorporated polymers in 8- and 18-h reactions (Fig. 4). Heparitinase I digested the incorporated products completely. When these digests were analyzed by high pressure liquid chromatography for glycosaminoglycan disaccharide component assay, only unsaturated GlcUA-GlcNAc disaccharide was detected (data not shown). Chondroitinase ABC, *Streptomyces* hyaluronidase, and sheep testicular hyaluronidase did not digest the polysaccharide products (data not shown). These results indicate that both products were HPR polysaccharide chains.

DISCUSSION

The K5 capsular polysaccharide synthesized by *E. coli* strain K5 consists of disaccharide repeating units of (-4 GclA β1–4 GlcNAc α1-), a backbone of HS. A gene cluster responsible for its synthesis encodes the following four proteins: KfiA, KfiB, KfiC, and KfiD. Of them, KfiA and KfiC were supposed to be GlcNAc-T and GlcA-T, respectively. Whereas recombinant KfiA was expressed, purified, and identified to be GlcNAc-T (14), the enzyme activity of purified KfiC had yet to be determined. Here, we have successfully expressed KfiC as a fusion protein with TF for the first time, and we have shown that it exerts GlcA-T activity in the presence of KfiA. Furthermore, we have demonstrated that both KfiA and KfiC form a complex, which exhibits polymerase activity to generate HPR chains. Our results provide mechanisms underlying biosynthesis of HPR in *E. coli* strain K5 and would lead to bioengineering of HS chains.

Although KfiC was speculated to have GlcA-T activity, the recombinant fusion protein TF-KfiC, by itself, had no GlcA-T activity. Addition of KfiA to the TF-KfiC reaction attained GlcA-T activity. Analysis using various mutants of D*X*D motifs confirmed the sequence of ^{79}DDD of KfiA as responsible for GlcNAc-T activity and that of ^{352}DAD of KfiC as responsible for GlcA-T activity in the crude cell lysate assays (12, 13). Whereas a mixture of intact KfiA with D352A KfiC mutant did not show GlcA-T activity, that of intact KfiC and D79A KfiA mutant did, indicating the GlcNAc-T catalytic site of KfiA is not necessary for the GlcA-T activity of KfiC. By interacting with KfiA, KfiC may change its conformation and acquire GlcA-T activity. In the presence of both UDP-GlcUA and UDP-GlcNAc as donors, the enzyme mixture efficiently polymerized HPR chains onto HPR oligo- and polysaccharides, suggesting that these enzymes are present in a complex.

His-KfiA Δ211–238 truncated mutant that lacks the C-terminal 28 amino acids had neither its own GlcNAc-T nor KfiC activation activities; however, the peptides at the C-terminal region indicated neither activation nor inhibition effects on GlcA-T. The interaction site of KfiA with KfiC is not limited to the C-terminal region, although it must be important for both GlcNAc-T and GlcA-T activities.

Four positions in the sequence of KfiA containing ionic amino acid clusters that may be concerned in the interaction were selected. The point mutants at N-terminal sites (K13A/K14A/E15A and E38A/E39A) exhibited GlcNAc-T activity as well as intact KfiA, although the mutants at C-terminal sites (E188A/K189A and K209A/K210A) had lower activity of GlcNAc-T than intact KfiA. These results suggested that the C-terminal site is responsible for the transferase activity of KfiA. In regard to activation activity for GlcA-T, the activity of three mutants (K13A/K14A/K15A, E38A/E39A, and E188A/K189A) was increased 1.5–2.2 times, and the activity of the point mutant replaced at the most C-terminal site (K209A/K210A) was decreased slightly. These positions were suggested not to be very important for demonstrating the activation of GlcA-T activity.

The shorter truncated KfiC mutants (Δ8–14, Δ8–39, and Δ8–49) showed GlcA-T activity with intact KfiA; however, the longer truncated mutants (Δ8–75, Δ8–150, and Δ8–225)

hardly had any activity. This strongly suggested that the amino acid sequence between 49 and 75 is essential for GlcA-T activity. However, it is not yet known whether the sequence in KfiC is necessary for its own GlcA-T activity or for the interaction with KfiA.

The lack of enzymatic activity of KfiC may be due to the presence of an inhibitory domain within the molecule, and the interaction of KfiC with KfiA may cancel out the action of such an inhibitory domain. However, all truncated mutants of KfiC that we examined represented no GlcA-T activity without the aid of KfiA. There is little possibility of this hypothesis.

GlcA-T activity shown by the interaction of KfiA and KfiC was reduced by increasing NaCl concentrations and abrogated at physiological concentrations. These observations indicate that KfiA and KfiC have a tendency to interact weakly with each other and that their stable association under physiological conditions requires other molecules. Previous studies revealed that KfiA, KfiB, and KfiC were localized in the cytoplasmic membrane and that KfiB is required for the association of KfiC with the membrane (13). Other K5 capsular molecules (KpsC, KpsD, KpsE, KpsF, KpsM, KpsS, KpsT, and KpsU) involved in the synthesis and attachment of phosphatidyl-KDO and exportation of the polysaccharide chains across the plasma membrane are known to be required for association of the K5 synthetic proteins (KfiA–D) with the membrane (7, 20). They may be involved in the stabilization and localization of the enzyme complex.

GlcA-T activity of the enzyme mixture at different ratios increased with additional KfiA up to a 1:1 molar ratio of KfiA and KfiC. It also represented an efficient HPR polymerization reaction of alternating GlcUA and GlcNAc transfer to HPR oligo- and polysaccharides. These results suggest that the two enzymes interact at an equimolar ratio. In contrast, reactions with different amounts of KfiC showed a similar level of GlcNAc-T activity of KfiA, consistent with the previous report (14).

Although the GlcNAc-T activity of KfiA was little affected by various pH and salt concentrations, the GlcA-T activity of the complex was largely affected. The presence of Mn^{2+} ion (2 mM) in the reaction buffer showed the highest activity of the GlcNAc-T and GlcA-T of KfiA and KfiC, like other glycosyltransferases (21). A higher concentration (20 mM) of $MnCl_2$ reduced the GlcA-T activity but not the GlcNAc-T activity, presumably due in part to instability of UDP-GlcUA at high $MnCl_2$ concentrations (22). The effects of various divalent metal ions on the enzyme activity were different between GlcNAc-T and GlcA-T, which may be due to differences of glycosylation reaction (α1–4, retaining reaction, or β1–4, inverting reaction) and substrate structure (GlcNAc or GlcUA) (23).

Many elongation enzymes for glycosaminoglycan biosynthesis are bifunctional glycosyltransferases. For example, animal species have EXT-1 and -2 for HS chain elongation (3), CSS-1/ChSy-1, CSS-2/ChPF, and CSS-3/ChSy-2 for chondroitin chain elongation (24, 25), and HAS-1, -2, and -3 for hyaluronan synthesis (26). Many types of bacteria also have dual action enzymes (27), such as HAS-A (*Streptococcus* sp.), pmHS1, -2, pmCS, and pmHAS (*Pasteurella multocida*), and K4CP (KfoC, *E. coli* strain K4) (28). The majority of the enzymes consist of two independent glycosyltransferase sites containing D*X*D motifs, except for HAS-1, -2, and -3 and HAS-A (class 1 hyaluronan synthase) (29). The crystal structure of K4CP revealed two distant active sites (30). PmHS-1 and -2 contain two glycosyltransferase sites at the N- and C-terminal regions similar to the active sites of KfiC and KfiA (31), and they have dual transfer activity to synthesize the HPR polymer (32). These dual action enzymes might be evolutionally derived by gene fusion from a pair of genes encoding single-action glycosyltransferases, gaining efficient polymerizing activity of heteropolysaccharides. It is intriguing that KfoC in the gene cluster of *E. coli* strain K4 contains both GlcA-T and *N*-acetylgalactosaminyltransferase sites and polymerizes a backbone of chondroitin chains itself (15), whereas in strain K5, the complex of KfiA and KfiC polymerizes HPR. Further studies remain to be performed to understand the molecular basis for their distinct catalytic mechanisms.

We revealed that HPR polymer synthesized by *E. coli* strain K5 serves as a good substrate for GlcNAc-T assay but not for GlcA-T assay. This was constantly observed in five different HPR samples purified by individual lots from *E. coli* K5 culture. Both GlcNAc-HPR substrates, prepared by removing a GlcUA residue at the nonreducing terminus of HPR with glucuronidase and by adding a GlcNAc residue at the nonreducing terminus of HPR with KfiA, were useful for GlcA-T analysis.

Our results clearly demonstrate that the complex of KfiA and KfiC is the core machinery of HPR biosynthesis. The three-dimensional structure of these glycosyltransferases and their complex remains to be determined. Further characterization of the enzyme complex containing other molecules such as KfiB would reveal detailed mechanisms of HPR polymerization. This study provides a useful tool to generate expected chain length of HPR, and it leads to preparation of different HS chains in length and structure by modification with HS sulfotransferases and HS C5-epimerase. Such defined HS chains would enable a detailed analysis on functions of HS and, in the future, could be used for clinical applications.

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