Molecular Cloning, Identification, and Sequence of the Hyaluronan Synthase Gene from Group A Streptococcus pyogenes*

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The hyaluronan (HA) synthase of Group A Streptococci has been identified by transposon mutagenesis and deletion analysis. The genes for the HA synthase and a recently identified UDP-Glc dehydrogenase (Dougherty, B. A., and van de Rijn, I. (1993) J. Biol. Chem. 268, 7118-7124) reside on a contiguous stretch of 3.2-kilobase pair DNA that can direct HA biosynthesis in Enterococcus faecalis and Escherichia coli as well as mutant Streptococcus (DeAngelis, P. L., Papaconstantinou, J., and Weigel, P. H. (1993) J. Biol. Chem. 268, 14568-14571). The synthase contains 395 residues (calculated Mr = 45,063) and migrates on SDS-PAGE with a molecular mass of 42 kDa. E. coli K5, which synthesizes UDP-glucuronic acid for production of its endogenous capsular polysaccharide, can make HA if it contains a plasmid encoding the intact 42-kDa protein. E. coli Sure or χ1448 cells containing the same construct, however, cannot produce HA since these strains cannot make both required sugar nucleotide precursors. The HA synthase is predicted to be an integral membrane protein with four membrane-associated helices, which is consistent with the location of the enzyme activity in Streptococci. There is significant homology between the HA synthase and the Rhizobium nodC gene product, an enzyme that synthesizes chitin-like oligomers. This is the first description of the molecular level of an enzyme shown to synthesize a glycosaminoglycan.

HA, a glycosaminoglycan composed of alternating GlcA and GlcNAc, has remained the focus of numerous inquiries since its discovery almost 6 decades ago (1). HA is a major constituent of the vitreous humor of the eye, synovial fluid, and extracellular matrices, and skin (2). The polysaccharide also interacts with various receptors and binding proteins that modulate cellular behavior such as migration, adhesion, and wound healing (2). Interestingly, HA is also found in the extracellular capsule of pathogenic Group A and C Streptococci (3). The bacterial capsule is a virulence factor that allows evasion of host defenses (4). The enzyme that polymerizes the polysaccharide is HA synthase. Although cell-free biosynthesis of HA was achieved over 3 decades ago (5) and HA synthase activity has been detergent-solubilized from plasma membranes of both eukaryotes and bacteria (6, 7), a functional enzyme has not yet been purified to homogeneity. The synthase is membrane-associated and utilizes UDP-sugar nucleotides at neutral pH in the presence of Mg2+ (5, 6).

A 52-kDa protein from Group C Streptococcus equisimilis was reported to be the synthase since antibody to it inhibited enzyme activity (5). This 52-kDa protein, however, was not shown to be biologically active, and the cloned gene did not confer HA biosynthesis to any organism (9). van de Rijn and Drake (10) used [3-H]-UDP-GlcA, to label proteins of 27, 33, and 42 kDa in both GAS and GCS. The incorporation of radiolabel was competed with excess UDP-GlcA, and GAS mutants lacking HA synthase activity did not display the same labeling pattern. Dougherty and van de Rijn recently presented two different models of the putative Streptococcus HA biosynthesis operon based on Tn mutagenesis data. The first model (11) invoked two oppositely translated ORFs (ORF A and ORF B), while the latter (12) described three ORFs (hasA, hasB, and hasC) in the same orientation; only ORF A (hasA) was shared between the two models. HasB was shown to be a 402-residue protein, UDP-Glc dehydrogenase, that produces one of the two sugar nucleotide precursors, UDP-GlcA, required for HA biosynthesis (12).

We recently reported that a 3.2-kb region of streptococcal DNA encoding two proteins of 42 and 45 kDa could functionally reconstitute HA biosynthesis in vivo in acapsular GAS mutants, Enterococcus faecalis or Escherichia coli (13). By deletion analysis, we showed that the 42-kDa protein was essential for HA biosynthesis activity. In this report, we identify the 42-kDa protein as HA synthase, the gene product of hasA. We also confirm that the 45-kDa protein is the UDP-Glc dehydrogenase.

EXPERIMENTAL PROCEDURES

Materials and Strains—Restriction and DNA modifying enzymes were from Promega unless otherwise noted. All other reagents were of the highest grade available from Sigma unless stated otherwise. Media reagents were from Difco. Cultures to be assayed for HA were grown using the dialysate from dialedyzed THY broth (i.e. nutrients <10-14 M). The mucoid GAS strain, S43/192/4, was obtained from the Rockefeller Collection (14). E. coli K5 (B18337-41) was obtained from I. Orskov and F. Orskov (Copenhagen, Denmark; Ref. 15). All other strains and plasmids used were described previously (13).

Tn Mutagenesis and Mutant Selection—Induction of mutagenesis was conducted by the method of O'Connor and Cleary (16) except that ovine hyaluronidase (Type V) was added to the GAS culture (0.2 mg/ml, 1 h at 37 °C) after overnight growth and used at a higher concentration (0.1 mg/ml) in the mating plate media. The Tn916 donor, E. faecalis CG110 (17), was mated on nitrocellulose filters (88 mm, 0.45 μm, Millipore Separation, Inc.) with strep' S43. The mating mixture was scraped off the filters with 0.4 ml of THY broth containing 1 mg/ml streptomycin and 5 μg/ml tetracycline. The nonmucoid mutant cells were then enriched over Percol (Pharmacia LKB Biotechnology Inc.) step gradients (13, 18). Acapsular (or hyaluronidase-treated) cells pelleted through 50% glycerol.
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Percoll, but mucoid cells float at the interface. After overnight outgrowth (50–70 μl of mating mixture/5 ml of double selective media with 5% serum in a 15-ml tube), the cultures were underlaid with 2 ml of 50% Percoll in water and centrifuged (3,000 × g for 10 min). The media, the cells at the interface, and most of the Percoll were removed by aspiration, and the cell pellet was then used to inoculate 5 ml of fresh double selective medium in a further round of growth for 16–18 h. A 0.2–0.61 gradient enrichment were performed. Portions of the final cell pellet were streaked on double selective plates containing 5% sheep blood and visually screened for candidate mutants of capsule biosynthesis: those with dry, discrete colonies versus wild-type wet, spreading colonies. The mutants were streak-purified and verified to be similar to wild-type S43 with respect to vigor, β-hemolysis, DNase secretion (using DNA from the mutants and transductants was cut with HindIII and analyzed by Southern blot analysis). Therefore, the cell pellet was then used to inoculate 5 ml of fresh double selective media.

The sequence obtained beyond the EcoRI site of pB3 (left site; Ref. 13) was used to screen two libraries (13) to obtain the intact 12-kb inserts. Southern analysis of HindIII digests of chromosomal DNA of the Streptococcus pneumoniae strain S43 (1448) or SOLR cells (Stratagne) with the Exassist helper phage (Stratagen). One clone, pB3, was analyzed by sequencing with Sequenase using the standard protocols. The phage selected from the AGEM library using HA3 were sequenced with another oligonucleotide (HA6; ATGGCTTGGCAGC), corresponding to the sequence found near the end of the pB3 insert, in order to obtain DNA adjacent to pB3.

Two positively hybridizing AGEM isolates, which formed small plaques and grew poorly in liquid lysates, were obtained. Large scale plate lysates with top and bottom agarose were needed in order to prepare their DNA (22). The two clones (λX1 and λX2 with 20- and 12-2 kb inserts, respectively) contained the same region of DNA as determined by direct sequencing of the A DNA (22). The filters were hybridized with end-labeled oligonucleotide (HA3; TGGCACAATATGTCAGCCC), in 1.8 × HB (1 mg/ml of probe/8 ml) with 1% sarcosyl, 0.5% nonfat milk at 42 °C for 3 h and washed with 0.5 × HB at the same temperature for 1 h. The plaques yielding the strongest signal were replated and rescreeened twice. Purified phage from a λZAP library were converted to plasmid form by coinfecntion of SURE or SOLLR cells (Stratagen) with the Exassist helper phage (Stratagen). One clone, pB3, was analyzed by sequencing with Sequenase using the standard protocols. The phage selected from the AGEM library using HA3 were sequenced with another oligonucleotide (HA6; ATGGCTTGGCAGC), corresponding to the sequence found near the end of the pB3 insert, in order to obtain DNA adjacent to pB3.

RESULTS AND DISCUSSION

The HA synthase gene of GAS was initially identified by its insertional mutagenesis. The bacteriophage λ25-transducing lysate (19) from one acapsular mutant (designated S43Tn7), which contained two Tn elements, transmitted the nonmucoid phenotype to three out of five transductants (Fig. 1). The nonmucoid transductants did not possess HA synthase activity or a capsule by light microscopy, but the mucoid transductants were equivalent to wild-type S43. HindIII digests of mutant S43Tn7 chromosomal DNA showed two bands migrating at 16 and 18 kb on agarose gels that corresponded to the higher Mr, bands detected by a Tn-specific probe on Southern blots of all Tn16 mutants (Fig. 1). These larger species are the result of adding 10 kb of Tn DNA to the S43 HindIII fragment at the insertion site. Since the Tn-tagged DNA from S43Tn7 was well resolved from the other HindIII fragments, it could be gel-purified.

The 16- and 18-kb chimeric fragment associated with the HA biosynthesis of various S43 strains using a Tn-specific probe (22) reveals that S43Tn7 (T) contains two Tn insertions (each Tn yields two bands due to an internal HindIII site). Transduction segregates the two Tns and produces nonmucoid (N, N') or mucoid (M, M') colonies (two independent clones of each are shown). Wild-type S43 (W) DNA does not hybridize with the probe. All the wild-type HindIII fragments detected with ethidium bromide (EB panel) migrate at ≤10 kb (S; a HindIII standards in kb). Therefore, the chimeric Tn-tagged fragments (marked with arrows) were purified and sequenced directly. An oligonucleotide probe specific for the HA biosynthetic locus was derived from the fragment marked with the star.
were present (Fig. 2) in agreement with the earlier minicell streptococcal DNA, two constructs were made that substantially truncated either HasA or HasB (Fig. 2). One plasmid, pPD41A5, was obtained using both the nested nuclease deletion set with the M13 vector primers and the functional plasmid with custom oligonucleotides. Two major ORFs (Fig. 3) were translated from the primary structure of a previously undescribed protein (Fig. 3). The deduced polypeptide contains 395 residues with a predicted molecular weight of 45 kDa. Sites for EcoRI and PstI (P), and EcoRV (R) are marked. The Tn insertion site was about 4 kb to the right of the E site on the wild-type map but the intervening chromosomal DNA was deleted in the S43Tn7 mutant (13). The various pPD41 deletion constructs are depicted (black lines) below the map.

Defect was therefore used directly as a template for sequencing reactions with a Tn-specific primer that reads outward from the Tn terminus and into the interrupted gene. An oligonucleotide (HA3), corresponding to a portion of the sequence of the interrupted gene from the 18-kb chimeric fragment, was used as a hybridization probe for screening wild-type S43 genomic DNA libraries in λ phage. An excised λZAP clone, pB3, containing a 5.5-kb EcoRI fragment was selected and studied further. However, Southern analysis utilizing various oligonucleotide probes to the sequence of pB3 revealed some discrepancies between the wild-type and Tn mutant genomes (e.g. HA16 hybridized to S43 but not S43Tn7, while HA3 hybridized to both; not shown). Therefore, a larger genomic fragment spanning the Tn-induced deletion (13) was obtained from the λEM library. After an extensive subcloning effort and subsequent exonuclelease III deletion, a 3.2-kb fragment of S43 DNA was identified as a locus that could direct HA biosynthesis (13).

The sequence of the complementing streptococcal DNA, the insert of pPD41Δ5, was obtained using both the nested nuclease deletion set with the M13 vector primers and the functional plasmid with custom oligonucleotides. Two major ORFs were present (Fig. 2) in agreement with the earlier minicell analysis (13). The sequence of the first ORF, hasA, reveals the primary structure of a previously undescribed protein (Fig. 3). The deduced polypeptide contains 395 residues with a molecular weight of 45.063. The 42-kDa protein observed by SDS-PAGE analysis of pPD41Δ5 minicells is assigned to be HasA because the pPD41Δ5 plasmid, missing about half of the hasA gene (Fig. 2), does not produce the 42-kDa species (Fig. 4). HasA is predicted to have a pl of 8.2 and to be an integral membrane protein due to four membrane-associated regions (three predicted transmembrane segments).

To identify the role of the two genes on the complementing streptococcal DNA, two constructs were made that substantially truncated either HasA or HasB (Fig. 2). One plasmid, pPD41Δ5 EcoRV, should produce the intact 45-kDa protein, HasB. The other, pPD41Δ5I, should make the intact 42-kDa protein, HasA. The pPD41Δ5 EcoRV construct, in which the truncated hasA gene produced a new 27-kDa species (instead of the 42-kDa protein) as determined in minicells (Fig. 4), did not confer the ability to produce HA in any host (Table I). Minicells containing pPD41Δ5I produced two nonvector-derived proteins: the intact 45-kDa protein, HasB, and a 29-kDa truncated version of HasB (Fig. 4). The deleted hasB gene product is predicted to be 23 kDa based on the sequence. When transformed into SURE or χ1448

Fig. 2. Schematic map of the HA biosynthesis locus and various plasmid constructs. A restriction map of the complementing region of S43 DNA, containing two substantial ORFs, is shown. The hasA and hasB genes are translated in the same orientation but in different reading frames. Sites for EcoRI (E), HindIII (H), ClaI (C), BglII (B), PstI (P), and EcoRV (R) are marked. The Tn insertion site was about 4 kb to the right of the E site on the wild-type map but the intervening chromosomal DNA was deleted in the S43Tn7 mutant (13). The various pPD41 deletion constructs are depicted (black lines) below the map.

Fig. 3. Nucleotide and deduced protein sequence of the E. coli minicell analysis of pPD41 deletion constructs. Minicells from χ1448 containing various plasmids were 5% labeled, and the proteins were separated on a 12.5% SDS-PAGE gel. This autoradiogram (10-h exposure) shows that when hasA or hasB genes are disrupted, the predicted proteins (HasA, filled arrow at 42 kDa; HasB, open arrow at 45 kDa) are likewise effected. The truncated versions of HasA (filled circle) and HasB (open circle) are also shown. The sequence of the hasA gene is also shown. The sequence of the hasA gene is also shown.
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**Table 1**

| Strain     | Plasmid   | HA* (ng/µl) | UDP-Glc DH1* (pmol/min/µg) | Protein† |
|------------|-----------|-------------|---------------------------|----------|
| SURE       |           | 0           | 3                         | -        |
| PD41Δ5     |           | 64          | 19                        | +        |
| PD41Δ7     |           | 0           | 7                         | +        |
| PD41ΔPstI  |           | 0.2         | 0.8                       | +        |
| PD41ΔEcoRV |           | 14          | 14                        | -        |
| x1448      | AT19      | 0           | 0.8                       | -        |
| PD41Δ5     |           | 21          | 17                        | +        |
| PD41Δ7     |           | 0           | 6                         | -        |
| PD41ΔPstI  |           | 0.6         | 2.5                       | +        |
| PD41ΔEcoRV |           | 10          | 10                        | -        |
| K5 (BI8337-41) | AT19 | 0 | 12 | - |
| PD41Δ5     |           | 253         | 12                        | +        |
| PD41Δ7     |           | 0           | 17                        | -        |
| PD41ΔPstI  |           | 49          | 13                        | +        |

*Spent culture medium and SDS cell extracts were pooled and assayed; values are normalized to 1 A260 unit of cells/ml.

†NADH production was measured in soluble cell extracts in the presence of UDP-Glc. There was no activity in the absence of UDP-Glc.

**Presence or absence of intact HasA (42 kDa) or HasB (45 kDa) gene product only in the presence of UDP-GlcA or UDP-Glc, respectively.**

**Table 1** HA production and UDP-Glc dehydrogenase activity in E. coli strains containing various pPD41 constructs.

**FIG. 5.** Sequence homology of HasA and NodC. The most conserved regions of the two proteins are shown with identical residues in boldface. These residues may be essential for activity. Several conservative substitutions are also present (e.g. D/E, K/R, or ST).

**synthase.** NodC possesses several stretches of residues that are identical or similar to the HA synthase (Fig. 5). Overall the two proteins are 30.6% identical. The hydropathy plots of the two proteins are comparable, including three predicted transmembrane segments in the same location near the carboxyl terminus (not shown). Other proteins with homology to HA synthase include DG42 from Xenopus laevis, yeast chitin synthase II, and an associated protein CSH2 (28). The 52-kDa protein described by Prehm and co-workers (8, 9) is not homologous to HasA.

**HasA and hasB are the only exogenous genes required to direct HA biosynthesis in most bacteria, due to the presence of one of the sugar nucleotide precursors of HA, UDP-GlcNAc, which is necessary for cell wall formation. In cells that make both UDP-GlcNAc and UDP-GlcA only HA synthase, the gene product of hasA, is needed to polymerize the HA polysaccharide.**

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