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Dendrimer-Enabled Modulation of Gene Expression in *Chlamydia trachomatis*

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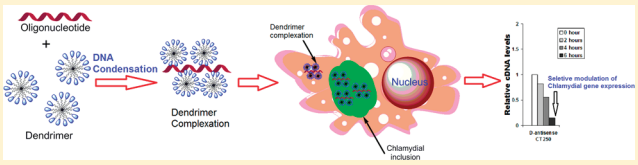
ABSTRACT: The obligate intracellular bacterium *Chlamydia trachomatis* is an important human pathogen. The genome of this organism is small but encodes many genes of currently unknown function that are thought to be involved in virulence. Lack of a system for genetic manipulation has been a key challenge to advancing the understanding of molecular genetics underlying virulence for this bacterium. We developed a dendrimer-enabled system for transformation of *C. trachomatis*, and used it to demonstrate the efficient and highly specific knockdown of transcript levels from targeted genes. Antisense, sense, and other control oligonucleotides targeting two sets of duplicated genes on the chlamydial chromosome were designed, commercially synthesized, and complexed with generation-4 polyamidoamine (PAMAM) dendrimers. The complexes were given to HEp-2 cell cultures infected for 16 h with *C. trachomatis* serovar K and then removed three hours later. Infected cultures were harvested 6 h after pulsing, and DNA and RNA/cDNA were prepared for assessment of transcript levels compared to those for the same genes in infected cultures, without dendrimer complexation. In all cases, the targeted gene complexed to dendrimer, but not its duplicate, showed up to 90% transcript attenuation. The duration of attenuation can be extended by repeated pulsing, and in some cases transcript levels from multiple genes can be attenuated in the same organism. This system will allow study of chlamydial gene function in pathogenesis, leading to more effective therapies to treat *Chlamydia*-induced diseases in a targeted manner.



KEYWORDS: *PAMAM dendrimers, chlamydial infection, pathogenesis, knockout, bacterial transformation, dendrimer−oligonucleotide complexes*

INTRODUCTION

Chlamydia trachomatis is an intracellular bacterial pathogen of humans. This organism is an important etiologic agent for genital infections in the United States and elsewhere.[1,2] Indeed, in the US chlamydiae constitute the most prevalent sexually transmitted bacterial infection, affecting more than 4 million people, and new genital chlamydial infections must be reported to the Centers for Disease Control and Prevention from all 50 states and the District of Columbia.[3] Importantly, genital chlamydial infections often engender severe sequelae, including reproductive problems in women and inflammatory arthritis in both genders.[2,4] Further, *C. trachomatis* also is the causative organism in blinding trachoma, which remains a significant source of treatable blindness in some underdeveloped nations.[2] Understanding the genetic functioning of this important organism can provide opportunities for treatment and prevention.

C. trachomatis undergoes a transcriptionally governed biphasic developmental cycle.[5,6] The cycle is initiated when elementary bodies (EB), the extracellular form of the organism, attach to a host cell. Once bound, EB are brought into a membrane-bound inclusion in the host cell cytoplasm. In the inclusion, EB undergo a development process resulting in production of reticulate bodies (RB), the metabolically active form of the organism. Each RB undergoes several cell divisions, and at the termination of the cycle RB dedifferentiate back to the EB form; these are released to the extracellular milieu by host cell lysis or exocytosis.[5] However, under some circumstances, including those relevant to disease sequelae, the cycle is arrested obviating production and release of new EB. This state is designated "persistence", and the arrest in the developmental cycle which engenders it is transcriptionally governed.[7−9]

The details of the genetic program that supports transition of actively infecting chlamydiae into the persistent infection state remain to be elucidated fully, but it is well established that other bacterial pathogens utilize a state of persistent infection to support their pathogenic processes, including *Mycobacterium tuberculosis* and others. Studies of the genetic basis underlying persistence for this organism showed that products from many genes of currently unknown function are required for the transit to, and maintenance of, persistence.[10] Our own studies have confirmed that this is also the case for *C. trachomatis*.[11] Importantly, DNA sequencing of the *C. trachomatis* genome

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identified just over 900 open reading frames overall, more than 200 of which specify products of unknown function.[12] While we have identified a number of those unknown coding sequences as being involved in chlamydial persistence, no practical system has existed for genetic manipulation of the organism to define details of that involvement.[13,14] Dendrimers are explored in this study to address this challenge, to deliver genes to chlamydial inclusions to enable a better understanding of chlamydial gene function.

Polyamidoamine dendrimers (PAMAM) are non-cytotoxic highly branched polymers, with a high degree of molecular uniformity and a narrow molecular weight distribution.[15,16] They are widely studied, with a large number of animal studies and preclinical trials currently underway in cancer therapy, imaging, and targeted delivery applications.[17−19] The high density of end groups on the dendrimer can produce a local environment that can modulate bacterial binding, intracellular transport and drug release. Apart from delivery of small molecules to specific sites, in the recent past PAMAM dendrimers have been efficiently used for intracellular transfection of oligonucleotides, plasmid DNA, peptides and biomacromolecules to cells.[20−25] They have the unique ability to bind to oligonucleotides, condense them and deliver them into a wide variety of cells.[26−28] PAMAM dendrimers are also used extensively for regulation of gene expression using antisense oligonucleotides.[22,29] When conjugated with peptide ligands, dendrimers can enable receptor-mediated gene delivery, leading to improved affinity and transfection efficiency and lower cytotoxicity.[30,31] For DNA delivery systems, the efficacy depends on the stability of the complex in the cytoplasm and the subsequent transport and release in the nucleus.[32,33] Dendrimers have been explored for the prevention of viral and bacterial infection, utilizing competitive surface binding interactions of dendrimers with host cells.[34−36] We have previously reported the intracellular uptake of PAMAM dendrimers by bacteria, and the subsequent prevention of preterm birth in pregnant guinea pigs.[37] We have also shown that these dendrimers can localize in the chlamydial inclusions inside host cells.[38] Building on this, we investigate whether these dendrimers can deliver single strand short oligonucleotides (ssDNA) into chlamydial inclusions, for selective modulation of gene expresssion in these pathogens.

A system for genetic manipulation of *M. tuberculosis* exists and is in use to define function for many genes of interest, including those encoding products of unknown function involved in persistence. In contrast, for *C. trachomatis* many groups have attempted to produce such a system for genetic manipulation, but success to date has been circumscribed. Recent reports describe systems that produce stable chlamydial transformants, but for several reasons the methods are impractical for regular use.[39,40] We developed a system for genetic manipulation of chlamydiae through delivery of various molecules to chlamydial inclusions using PAMAM dendrimers.[38] Initial characterization of that system showed that fluorescently labeled dendrimers accumulate quickly within *Chlamydia*-infected host cells in culture, concentrate within cytoplasmic inclusions containing the organism, and deliver antibiotics safely to the inlusions for release, and that exposure of infected or uninfected host cells to dendrimers does not produce significant stress in those cells.[38] We further demonstrated that the complexes deliver modifying DNA to the inclusions, including plasmids, and that such plasmids are maintained and expressed over subsequent passaging of the transformants.[41] In this report, we describe further development and characterization of this practical dendrimer-based system for the efficient introduction of modifying nucleic acids into growing *C. trachomatis* in cultured cells, and we demonstrate the specific knockdown of gene expression for any targeted coding sequence.

MATERIALS AND METHODS

Culture of *C. trachomatis*. *C. trachomatis* serovar K was used to infect eukaryotic host cells in culture in all experiments given here. That strain was maintained in, and cultured for experiments in, the human epithelial cell line designated HEp-2 as described.[7,8] A multiplicity of infection (MOI) of 2:1 was employed unless otherwise noted. Maintenance of host cell lines and preparation of chlamydiae for experiments were done as described.[8]

Oligonucleotides. Sense, antisense, scrambled DNA sequence, and other oligonucleotides used in these studies were designed by HCG and synthesized commercially (Integrated DNA Technologies, Coralville, IA, USA) (Table 1).

Table 1. Sense, Antisense, and Other Oligonucleotides[a]

oligonucleotide	sequence	no. of bases
CT275 antisense	5′-CCA CGT GCT GCA ATC ATT G-3′	19
CT275 sense	5′-CAA TGA TTG CAG CAC GTG G-3′	19
CT110 sense	5′-TAC AAC GAA GAA GCC AGA AAG AA-3′	23
CT110 antisense	5′-TTC TTT CTG GCT TCT TCG TTG TA-3′	23
CT334 sense	5′-TCT TCA AGA AGG TAC CGC C-3′	19
CT334 antisense	5′-TGG CCA AGA ATC TCG GAA-3′	18
CT250 sense	5′-TGC GAG CTT GGG AAG AGT T-3′	19
CT250 antisense	5′- AAC TCT TCC CAA GCT CGC A-3′	19
CT scrambled	5′-ACG TCT ACG TAC TAG TAC T-3′	19

[a]The amount was 1 μmol in each case.

Preparation of Dendrimer/Oligonucleotide Complexes. Amine-terminated generation-4 PAMAM dendrimers (G4-NH_2) (Dendritech, Midland, MI, USA) were complexed with oligonucleotides of various DNA sequences through addition of appropriate amounts of DNA in sterile deionized water. G4-NH_2 dendrimers have ∼64 primary amine groups (N) which can form complexes through electrostatic interactions with phosphate groups (P) of the DNA, with ratios of 4 and 8 (i.e., N/P ratio = 4, 8 and 16). After addition of the oligonucleotide, the mixture was kept at room temperature for 1 h and then centrifuged for 30 min. An aliquot was removed, and the complex was resuspended in the same volume of sterile deionized water. In control studies, it was determined that dendrimer/oligo complexes with N/P = 8 provided better experimental results for transfection over those with N/P = 4 and 16. Thus all experiments described here utilized complexes with N/P = 8.

Dynamic Light Scattering and Zeta Potential Measurements. The sizes of the complexes were measured by dynamic light scattering at 633 nm (DLS, Zetasizer Nano ZEN 3600, Malvern Instruments Ltd., Worcestershire, U.K). The samples (10 μg DNA equivalent in 1000 μL) were dissolved in DI water (18.2 Ω) and filtered using AccuSpin Micro 17/17R ultracentrifuge. Particle sizes were determined at 25 °C with a scattering angle of 173°. Zeta potentials were calculated using

the Smoluchowski model. Measurements were performed in triplicate in aqueous solution.

Transformation of *C. trachomatis*. HEp-2 cells were used as eukaryotic host for all experiments. Cultures with nearly confluent monolayers of these cells were treated in one of four ways: mock infection, mock pulsing (dendrimers + oligonucleotide mixture, not complexed), and complexed dendrimer/oligonucleotide at 0.2 μg and 1 μg. Dendrimers and dendrimer/DNA complexes were suspended in Iscove's modified Dulbecco's medium (IMDM) without FBS or glutamine. In most experiments, treatment of dendrimer/oligo complex at 1 μg proved optimal, and data reported here include primarily those from the 1 μg treatment. Uncomplexed dendrimers/oligonucleotides in IMDM, complexed dendrimers/oligonucleotides in IMDM at the two concentrations, or IMDM alone was added to the cell cultures at 16 h postinfection at 37 °C. At 19 h postinfection, fresh medium with glutamine and FBS and containing glucose (10 mg/mL) and cycloheximide (1 mg/mL) was added. Cultures so treated were harvested at 24, 36, and 48 h postinfection for analyses. For one set of experiments, infected HEp-2 cells were given the dendrimer/oligonucleotide complex at 16 h postinfection as above, then again at 22 h postinfection, as a test of extending the transcriptional attenuation effect. All experiments described were approved by the Wayne State University Biosafety Committee.

Preparation and Analyses of Nucleic Acids. Total nucleic acids were prepared for experimental purposes from infected and uninfected HEp-2 cell cultures using the hot phenol method, as described.[7,8,14] From those preparations RNA/cDNA for transcript analyses was prepared as described.[7] Real time RT-PCR analyses were done as described, using an Applied Biosystems model 7500 machine and the primer systems listed in Table 2.

Table 2. Primers Used for Real Time RT-PCR Analyses

primer	sequence
CT110 us	5′-TCACTCTAGGCCTAAAGGACG-3′
CT110 ds	5′-TCATGTTTGTCGGCAAGCTC-3′
CT187 us	5′-TCTTCATACGATTGAGACACC-3′
CT187 ds	5′-GTGTTATTCTATCGGCCTCAT-3′
CT250 us	5′-TATGCTCTTCGAACCAAGTT-3′
CT250 ds	5′-GCGAGCTTGGGAAGAGTT-3′
CT275 us	5′-CTTCTCGTCGACGATATCCA-3′
CT275 ds	5′-AAGTGGATCAGAGTTTCGAAG-3′
CT334 us	5′-GTCTGAATCGCGCAGCA-3′
CT334 ds	5′-GGCTCTTGATCTTGTGTAGGA-3′

Additional Methods. Western blotting was done as described.[42]

RESULTS

Preparation and Characterization of the Dendrimer–Oligonucleotide Complexes. Particle size and zeta potential of dendrimer–oligonucleotide complexes in N/P ratios (4, 8, and 16) are shown in Table 3. The size of the free dendrimer (G4-NH_2) was ∼4 nm, and it was not possible to measure the size of the oligonucleotides due to the low molecular weight. Out of these three N/P ratios, particle size was the smallest for N/P = 8 (∼100 nm), suggesting that the complexes of dendrimer and oligonucleotides were compact and stable. At this N/P ratio, we estimate that there are 2–3 dendrimers per one molecule of the oligonucleotide. When N/P was 4, the size of the particle was bigger, suggesting a less compact structure. The particle size was even bigger at N/P = 16. At this ratio, ∼5–6% of the dendrimer was free, suggesting that dendrimer coverage on the oligos may have reached its saturation. The zeta potential of G4-NH_2 was +19.5 mV, and that of all the free oligonucleotides was in range of −16 to −18 mV. The zeta potential values of the complexes were in the range of +30 to +45 mV. The bigger particle size suggested a lack of a compact structure, and a somewhat weaker interaction. The particle size was the smallest for N/P = 8, and the zeta potential was more positive than those of N/P = 4. Therefore, we used this N/P ratio in all our studies. The size of the resultant complexes and the zeta potentials were comparable to those used in the literature for oligonucleotide and siRNA complexes.[24,25,27,43]

Targeted Knockdown of Chlamydial Gene Expression. The first question to be addressed was whether we could attenuate the expression of a targeted gene on the chlamydial genome in a specific and efficient manner. DNA sequencing of the *C. trachomatis* chromosome identified two genes specifying the dnaA protein, one designated Ct250 in the genome sequence and the second designated Ct275.[12] We first targeted Ct250. 21-mer oligonucleotides of sense, antisense, and scrambled DNA sequence were designed, synthesized, and complexed to the G4-PAMAM dendrimers. One microgram of the dendrimer–oligo complex was added to HEp-2 cell cultures infected for 16 h with K serovar *C. trachomatis*; cultures so treated were harvested at 2, 4, and 6 h postpulsing, and RNA/cDNA was prepared from each for real time RT-PCR. Neither the sense nor the scrambled DNA sequence oligonucleotides nor the free dendrimers affected transcript levels from Ct250 at any time assessed, as shown in Figure 1A. However, the dendrimer–antisense 21-mer attenuated mRNA levels from Ct250 at 2, 4, and 6 h, at which time attenuation had reached about 90%. The level of attenuation increased gradually from 2 to 6 h, suggesting that the time scale of transport and release into the inclusions is several hours. The concentration of G4-NH_2 used in these studies is on the order of 1 μg/mL, well below where cell cytotoxicity was observed.[41] Importantly, this effect was specific for Ct250, since mRNA levels from dendrimer–Ct275 were unaffected in cells given the antisense oligonucleotide targeting Ct250. Control experiments, including one in which noncomplexed dendrimers and antisense oligonucleotides were added together to infected cells, showed no transcript attenuation from Ct250; transcripts from several other genes were unaffected as well (data not shown). The converse experiment using dendrimer/oligonucleotides targeting the Ct275 gene demonstrated that transcripts from this coding sequence also could be powerfully and specifically attenuated without affecting transcript levels from Ct250 (Figure 1B). Thus, complexes of dendrimers with antisense oligonucleotides, but not complexes prepared with various control oligonucleotides, strongly and specifically attenuate transcript levels from targeted genes.

Transcriptional Attenuation by Dendrimer/Antisense Oligonucleotides Is Not Specific to Ct250 and Ct275. To be certain that the powerful and specific transcriptional attenuation seen in the experiments above could be applied to other targeted genes in the chlamydial genome, parallel experiments were done targeting another two-gene set. As with the dnaA-encoding genes, the *C. trachomatis* genome includes two genes encoding the dnaX protein, one designated Ct187 and the other designated Ct334 in the genome sequence.[12] Transformation of infected HEp-2 cell cultures with dendrimers

Table 3. Size and Zeta Potential of Dendrimer−ssDNA Complexes

	size (nm)			zeta potential (mV)		
samples	$N/P = 4$[a]	$N/P = 8$	$N/P = 16$	$N/P = 4$	$N/P = 8$	$N/P = 16$
G4-NH2 (D)		3.9			19.5	
D−Ct334-sense	124.9	104.4	143.2	46.1	36.9	44.1
D−Ct334-antisense	123.5	93.2	118.1	27.3	40.5	43.8
D−Ct250-sense	119.2	97.8		38.2	47.3	
D−Ct250-antisense	115.9	101.6		35.9	42.5	
D−Ct275-sense	129.8	94.7		29.8	36.3	
D−Ct275-antisense	119.2	98.3		33.4	39.1	
D−Ct110-sense	122.4	113.2		32.9	38.6	
D−Ct110-antisense	114.3	99.2		31.8	40.2	
D−Ct-scrambled	117.7	99.1	145.2	39.1	34.8	43.2

[a] N = number of primary amine groups; P = number of phosphate groups.

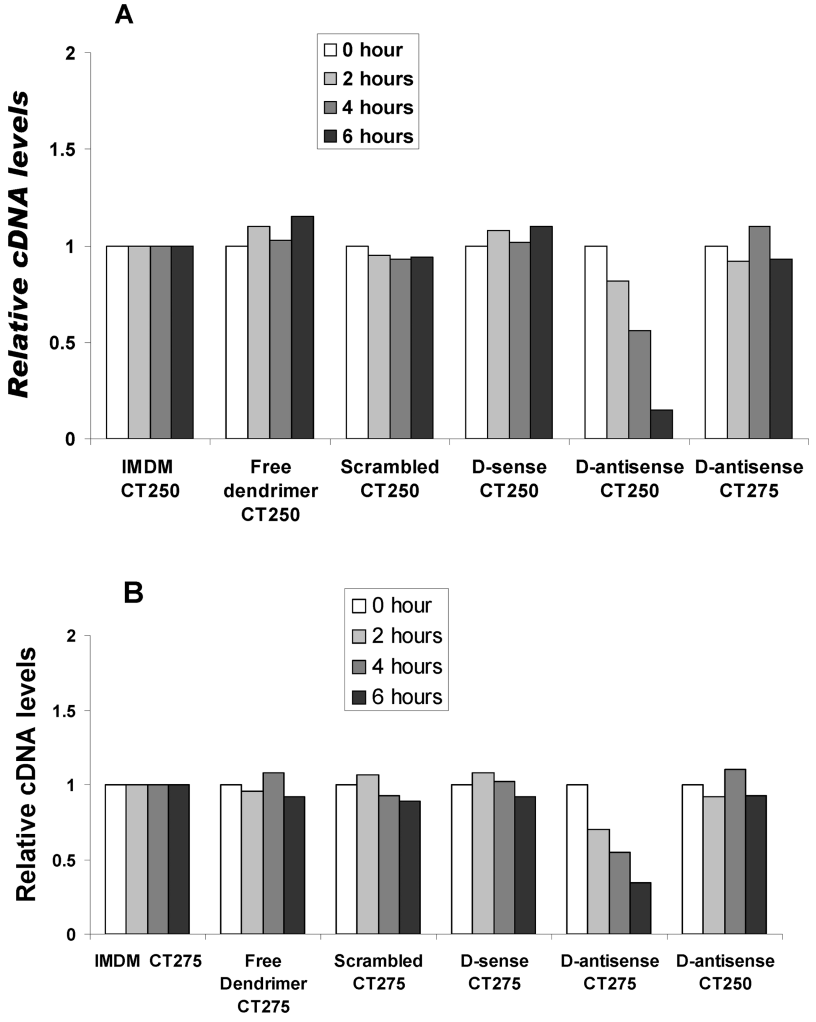

Figure 1. Quantitative assessment of transcript levels from the *C. trachomatis* Ct250 gene in infected HEp-2 cells given complexes of dendrimers with oligonucleotides ($N/P = 8$) of various DNA sequences (A) or the Ct275 gene (B). Nearly confluent layers of the host cells were infected at MOI 1:1 in both experimental sets, and at 16 h postinfection 1 μg of complexed G4/oligos was added to the medium. Cells were harvested at 2, 4, and 6 h after addition of complexes, and RNA/cDNA was prepared for real time RT-PCR assessment of relative transcript levels. Normalization to chlamydial 16S rRNA, data indexed to values in infected cells not given complexes. Data represent assays done in triplicate twice for each of two independent experiments. Note that while the antisense oligo targeting the Ct250 gene attenuated mRNA produced from that gene, transcripts produced from the other copy of the *dnaA* gene (Ct275) were unaffected and vice versa (Ct250).

complexed with sense, antisense, or scrambled DNA sequence oligonucleotides showed that specific transcript attenuation identical to that shown at Ct250 was achieved for Ct344 using the antisense complexes but not the control complexes (Figure 2); the other *dnaX,* Ct187, was unaffected. We also have done the converse experiment for knocking down Ct334 without

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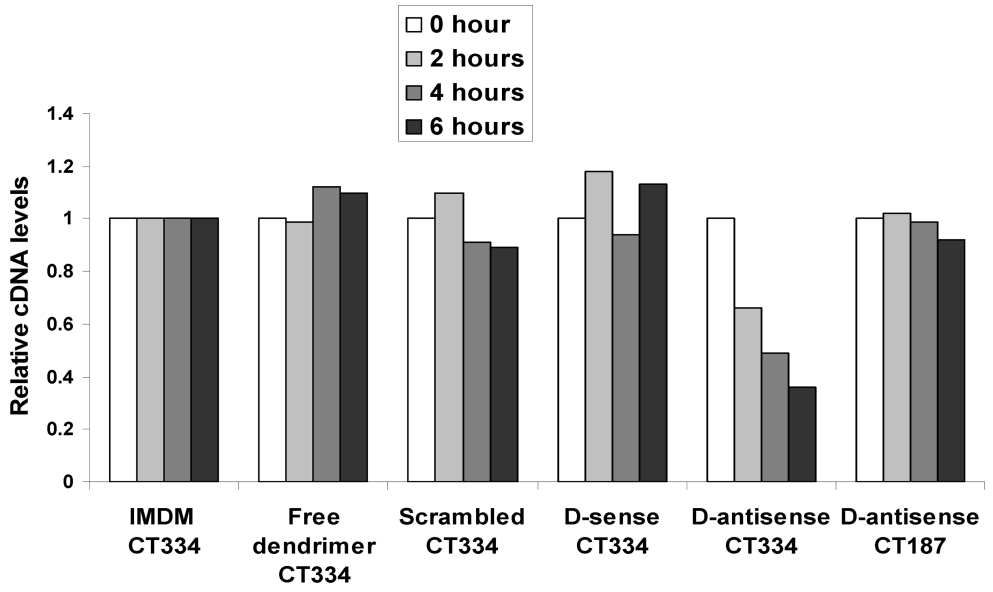

Figure 2. Quantitative assessment of transcript levels from the *C. trachomatis* Ct334 gene in infected HEp-2 cells given complexes of dendrimers with oligonucleotides ($N/P = 8$) of various DNA sequences. Nearly confluent layers of the host cells were infected at MOI 1:1 in both experimental sets, and at 16 h postinfection 1 μg of complexed G4/oligos was added to the medium. Cells were harvested at 2, 4, and 6 h after addition of complexes, and RNA/cDNA was prepared for real time RT-PCR assessment of relative transcript levels. Normalization to chlamydial 16S rRNA, data indexed to values in infected cells not given complexes. Data represent assays done in triplicate twice for each of two independent experiments. Note that while the antisense oligo targeting the Ct334 gene attenuated mRNA produced from that gene, transcripts produced from the other copy of the *dnaX* gene (Ct187) were unaffected.

affecting mRNA levels from Ct334 (not shown). Thus transcriptional attenuation achieved by use of dendrimer/antisense oligonucleotide complexes is not specific for one gene set.

Duration of Transcriptional Attenuation Achieved by Dendrimer/Antisense Oligonucleotide Complexes. An important issue relates to transcript attenuation *via* antisense oligonucleotide knockdown centers on the duration of the effect. In extended repeats of the experiments targeting the Ct250 gene, we found that the level of that attenuation at 12 h postpulsing was about 40%, and by 24 h postpulsing it had declined to only about 10%. We then repeated these experiments targeting Ct250 as outlined above, but at 22 h postinfection (i.e., 6 h after the initial pulsing of infected cells with dendrimer/antisense oligonucleotide complexes) we pulsed again with the complexes. Cultures so treated were then harvested for transcript analyses at 28 h postinfection. The data displayed in Figure 3 demonstrate that a high level of transcript attenuation can be maintained by simply repeating the pulsing of infected cultures with the antisense complexes. See the Discussion for more regarding this issue of duration of knockdown.

Transcriptional Attenuation from Multiple Genes Using Dendrimer/Antisense Oligonucleotides. We next asked whether two chlamydial genes could be targeted for knockdown in infected cells. Equimolar amounts of antisense oligos targeting Ct250 and Ct334 were complexed at the same time to dendrimers, as were equimolar amounts of sense oligos and DNA sequence-scrambled oligos targeting the same genes. K serovar-infected HEp-2 cell cultures were pulsed with these several complexes at 16 h postinfection as above and then harvested at 6 h postpulsing for transcript analyses. As shown in Figure 4, mock-pulsing/pulsing with the combined sense oligos produced no lowering of transcript levels from Ct250 or Ct334; pulsing with scrambled sequence oligos gave similar results (not

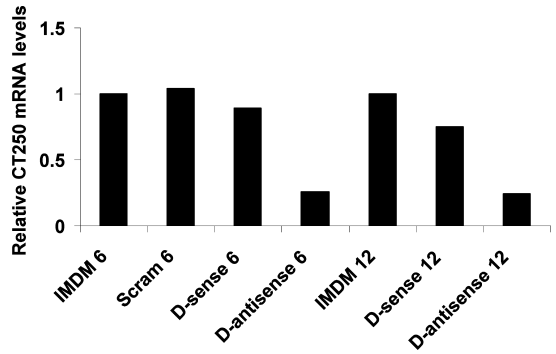

Figure 3. Relative transcript levels from the Ct250 gene in infected HEp-2 cells given complexes of G4 dendrimers with oligonucleotides of various DNA sequences twice. Nearly confluent monolayers of the host cells were infected at MOI 1:1 as in Figure 1, and at 16 h postinfection 1 μg of complexed G4/oligos was added to the medium. At 6 h postaddition of complexes, one set of cultures was harvested for RNA/cDNA preparation and real time RT-PCR assessment of relative transcript levels. Identically treated cultures were pulsed again with 1 μg of oligo–dendrimer complexes 6 h after the first pulsing, then harvested 6 h later (i.e., 12 h after the first pulsing), and again prepared for real time RT-PCR assessment of relative transcript levels. Normalization to chlamydial 16S rRNA, data indexed to values in infected cells not given complexes. Data represent assays done in triplicate twice for each of two independent experiments. The antisense oligo complexes targeting the Ct250 gene attenuated mRNA produced from that gene at 6 h after each pulsing.

shown). However, pulsing infected cells with the combined dendrimer–antisense oligo preparation congruently attenuated transcripts from both genes by about 70%. As with the single-targeting experiment shown in Figure 3, that attenuation had fallen to 40% by 12 h, and 10% by 24 h, postpulsing; repulsing with antisense complexes as above yielded an about 80% transcript attenuation from both genes 6 h later (not shown).

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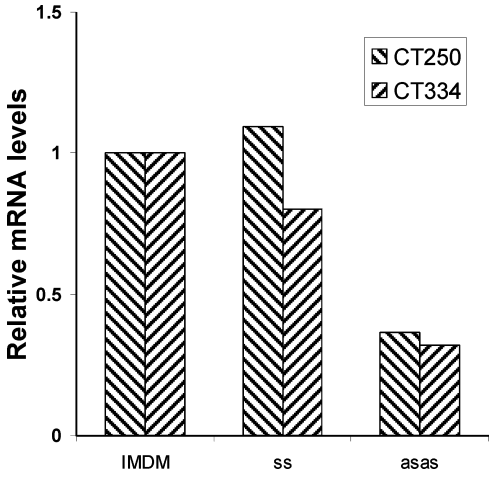

Figure 4. Transcripts from the *C. trachomatis* Ct250 and Ct334 genes in infected HEp-2 cells given complexes of dendrimers with oligos of various DNA sequence. HEp-2 cell cultures were infected at MOI 1:1, and at 16 h postinfection dendrimer/oligo complexes were added to the medium, then removed after 1 h. Cultures were harvested at 6 h postpulsing, and RNA/cDNA was prepared for real time RT-PCR assay of relative transcript levels. Normalization to chlamydial 16S rRNA, data indexed to values in infected cells not given complexes. IMDM, mock-pulsing with medium; ss, combined sense oligo/dendrimer complex; asas, combined antisense oligo complex.

Specific knockdown of two chlamydial genes is thus possible using this system.

Translation Products from Genes Whose Transcripts Have Been Attenuated Are Congruently Lowered. The purpose of knockdown experiments targeting any given gene is to attenuate the protein product from that gene. However, attenuation of transcripts as shown above does not prove that the relevant translation product is equivalently lessened. We therefore assessed knockdown from a chlamydial gene for which a high quality and well-characterized monoclonal antibody exists, in order to assess the protein product by Western blot analysis. The genome sequence for *C. trachomatis* identified three hsp60-encoding genes. The authentic hsp60 gene, *groEL*, which had been cloned and studied during the 1980s, was designated Ct110 in the genome sequence, and it, like the *Escherichia coli* and other versions, exists in an operon with *groES*.[12] The additional chlamydial hsp60 genes are designated Ct604 and Ct755; neither of the latter exists in an operon, and no gene resembling *groES* is found near either; these genes are not identical in DNA sequence to Ct110 or to one another. We prepared sense, antisense, and scrambled DNA sequence oligonucleotides targeting Ct110, complexed them to dendrimers, and transformed them into infected HEp-2 cells 16 h postinfection. Six hours after pulsing, we harvested cultures for analyses. We were somewhat concerned with lethality if Ct110 transcripts were too severely attenuated (see Discussion), and thus we pulsed with lower levels of dendrimer/antisense oligonucleotide complexes than used in experiments targeting Ct250, Ct187, and others. The data given in Figure 5A demonstrate that Ct110 transcript levels were attenuated by 49% in this experiment. Importantly, the Western blot data given in Figure 5B confirm that the translation product from the attenuated mRNA level was attenuated in rough proportion to that of its encoding messenger. However, we note that because transformation efficiency probably is not 100% in these experiments, the relative attenuation in

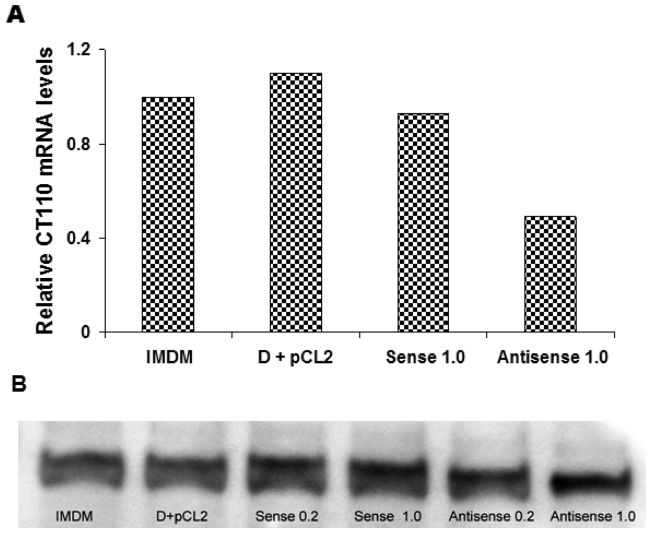

Figure 5. (A) Relative transcript levels from Ct110 in infected HEp-2 cultures given various control preparations (IMDM, free dendrimer, others not shown), and those given 1 μg of dendrimer−sense and −antisense oligo complexes. The only preparation to elicit attenuation of Ct110 mRNA levels was the antisense−dendrimer complex. Determinations done by real time RT-PCR, normalization to 16S rDNA. Data represent samples run independently twice, each time in triplicate. Standard error shown. (B) Western analysis of Ct110 protein in chlamydiae transformed with control and antisense oligos complexed or not to dendrimers. Antisense complexes elicited a 43% attenuation of protein level at 1 μg used. At 0.2 μg, attenuation was 15%. The monoclonal antibody used was a gift of Dr. G. I. Byrne.

translation product level is under-represented in the Western data given in Figure 5B. In future immunohistochemical studies, we will assess attenuation of this and other translation products following antisense-based knockdown using fluorescently labeled antibodies to visualize the specific targeted gene product within chlamydial inclusions in infected cultured host cells. Regardless, it is clear that knockdown of the transcript from *groEL* results in restricted levels of the encoded protein.

DISCUSSION

The genome of *C. trachomatis* is relatively small, specifying only about 900 open reading frames on a chromosome of 1 mbp. However, that genome includes more than 200 genes encoding products of unknown function.[12] Many, and perhaps most, of these unknown gene products are suspected to be involved in the pathogenic processes engendered by chlamydiae, as well as in the transition to/maintenance of the persistent infection state.[2,9,14] Importantly, to date elucidation of the functions of these important proteins has been difficult because no reliable and practical system for genetic manipulation of chlamydiae has been available. Development of such a system has been a goal of the *Chlamydia* research community for three decades, but to date progress has been relatively modest. One recently published transformation system for chlamydiae utilized an approach involving electroporation of DNA constructs into tissue culture cells infected with *Chlamydia psittaci*, a bird pathogen.[39] While it was the first published genetic manipulation system for chlamydiae able to produce reasonably stable transformants, the method requires large quantities of transforming DNA to produce low numbers of them and thus is cumbersome and impractical to use. The most recent genetic system described for chlamydiae provides significant improve-

ment on ease and reliability for transformation over the previous system, but selection over several passages is required to produce a clonal population of transformants.[40] Neither of these publications described a system for the gene-specific attenuation of expression in growing chlamydiae, however.

As described in this report, we have taken a nanotechnology-based approach to develop a transformation system for *C. trachomatis*, one that uses dendrimers as vehicles to deliver modifying nucleic acids to this organism as it is growing within its host cell inclusions. In another context, we demonstrate that dendrimers can be utilized to insert a plasmid into a plasmid-less strain of *C. trachomatis*. Transformants of *C. trachomatis* given the plasmid retain it through multiple passages, and the coding sequences specified on it are expressed; further, the system does function to introduce plasmid DNA into a related chlamydial species, in this case *Chlamydia pneumoniae*, even though that DNA is not normally present in that species.[41] In the present study we extend the utility of this dendrimer-based system to knockdown of gene expression in metabolically active chlamydiae. The system clearly is able to attenuate expression from targeted genes without affecting transcript levels from closely related or other genes. The attenuation can be maintained over extended periods by repeated pulsing of the *Chlamydia*-infected cells with the dendrimer−antisense complexes, and multiple genes can be targeted for knockdown in the same infected cell. Importantly, this dendrimer-enabled transformation system involves no electroporation or other unusual manipulation(s) of infected cell cultures, uses small amounts of oligonucleotide complexed to dendrimers, and in our hands is reliable for regular use. The system appears to be efficient, although we have not addressed this issue in a quantitative fashion as yet. Thus, this system for insertion of antisense oligonucleotides into chlamydiae will be useful for investigation of currently unaddressable questions regarding gene function in these organisms, especially when employed in concert with the ability to insert plasmids into these organisms.

It is important to note that initial characterization experiments for this genetic manipulation system using dendrimers with various species of DNA or antibiotics complexed to them showed no significant effects on chlamydiae or on their infected host cells. Specifically, introduction of dendrimers to infecting chlamydiae did not elicit persistent infection, nor could we identify any significant transcriptional response to dendrimer addition in infected or uninfected host cells.[38] In our studies, only a low MOI is used for infection of cultured host cells, and this generates an overall infection rate of some 30−50% in each culture. In control experiments with fluorescently labeled dendrimers, we were able to identify those dendrimers in both uninfected and infected cells. However, in infected cells the labeled dendrimers were concentrated somewhat in the chlamydiae-containing inclusions, an observation consistent with those of our earlier study.[38] We do not understand how or why this is the case, but we are investigating mechanisms by which this may be accomplished. In its current form, the transformation system includes no means to target dendrimer−DNA complexes specifically and solely to infected cells, and we are exploring various strategies to overcome this limitation.

The data presented in our other publications demonstrate that we have developed a system by which we can transform *C. trachomatis* and *C. pneumoniae* to insert plasmids to alter gene expression in this organism or antibiotic molecules to treat such infections.[38,41] The system allows the insertion of altered (mutated) coding sequences on the common chlamydial plasmid, or insertion of an exogenous gene under control of a chlamydial promoter. It also supports insertion of antibiotics to treat the infection. Importantly, the data presented here expand those initial observations to include the specific attenuation of transcript production from targeted genes on the chlamydial genome. This is a knockdown system rather than a knockout system, which is of significant advantage in a microbial system with such a small genome. That is, knockout of any given coding sequence, especially perhaps those specifying products of currently unknown function, may well engender lethality in such a small genome. The system described here obviates this issue by allowing a knockdown of gene expression from targeted coding sequences, rather than knocking them out. It will be important, however, to develop the knockdown system further so as to be able to control in a specific fashion the relative level of transcript attenuation from any given targeted gene or genes. Moreover, the relatively limited duration of gene expression is an avenue for significant improvements, and we thus consider it imperative to develop means to control the release of antisense oligonucleotides from the dendrimer−DNA complexes so as to extend the effect of transcript attenuation without repeated pulsing. Finally, while we have demonstrated that knockdown of expression from multiple chlamydial genes is possible, we do not know whether the system will be usable to attenuate in a specific manner both chlamydial and host cell gene expression. We are in the process of addressing both these issues.

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