Cloning and Characterization of RNA Polymerase Core Subunits of *Chlamydia trachomatis* by Using the Polymerase Chain Reaction

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Taking advantage of sequence conservation of portions of the α, β, and β' subunits of RNA polymerase of bacteria and plant chloroplasts, we have designed degenerate oligonucleotides corresponding to these domains and used these synthetic DNA sequences as primers in a polymerase chain reaction to amplify DNA sequences from the chlamydial genome. The polymerase chain reaction products were used as a probe to recover the genomic fragments encoding the β subunit and the 5' portion of the β' subunit from a library of cloned murine *Chlamydia trachomatis* DNA. Similar attempts to recover the α subunit were unsuccessful. Sequence analysis demonstrated that the β subunit of RNA polymerase was located between genes encoding the L7/L12 ribosomal protein and the β' subunit of RNA polymerase; this organization is reminiscent of the *rpoBC* operon of *Escherichia coli*. The *C. trachomatis* β subunit overproduced in *E. coli* was used as an antigen in rabbits to make a polyclonal antibody to this subunit. Although this polyclonal antibody specifically immunoprecipitated the β subunit from *Chlamydia*-infected cells, it did not immunoprecipitate core or holoenzyme. Immunoblots with this antibody demonstrated that the β subunit appeared early in infection.

Chlamydia trachomatis is an obligate intracellular parasite of eucaryotic cells (for reviews, see references 3, 26, and 27). This medically important gram-negative bacterium causes an array of ocular and genital disorders which rank among the most prevalent diseases of humans. Chlamydiae display a complex life cycle involving the sequential alternation of two different morphologic forms, the elementary body (EB) and the reticulate body (RB). The life cycle commences when the EB, the spore like metabolically inactive extracellular form, is taken up by the host eucaryotic cell. Upon binding to the host cell membrane and subsequent internalization into a host-derived endosome, the EB undergoes a striking morphologic transformation into the intracellular vegetative RB. The RB replicates by binary fission 100- to 1,000-fold while enclosed within this vacuole in the host cell cytoplasm. The newly replicated RBs subsequently redifferentiate into EBs that are released from the host cell, completing the intracellular life cycle.

Chlamydial development proceeds according to a strict program which clearly reflects the temporally regulated activation of specific sets of genes and at least superficially resembles the life cycle of the sporulating bacterium *Bacillus subtilus* (14, 15). The molecular basis of this developmentally regulated gene expression in chlamydiae is largely undefined, owing chiefly to the lack of convenient systems for gene transfer into this organism and to the paucity of information about the nature of the signals and machinery that govern chlamydial gene expression.

A major focus of our research has been to elucidate the *cis* elements and *trans*-acting factors that underlie the regulation of gene expression during this life cycle. In earlier studies, we and others have shown that chlamydial promoter sequences appear to be different from those of other procaryotes (9, 24, 29; J. Engel and D. Ganem, in *Immune Recognition and Evasion: Molecular Aspects of Host-Parasite Interaction*, in press), and in fact, no chlamydial promoter

tested so far functions properly in *Escherichia coli* (24; Engel and Ganem, in press).

A fuller understanding of chlamydial gene regulation will require a more detailed characterization of chlamydial RNA polymerase, the central component of the transcriptional apparatus. Eubacterial RNA polymerases are multisubunit enzymes composed of α, β, β', and σ subunits (reviewed in reference 11). The core enzyme, $\alpha_2\beta\beta'$, is a nonspecific DNA-binding protein. Holoenzyme, formed by the association of the σ subunit with core enzyme, has the property of sequence-specific DNA recognition, permitting the specific binding of RNA polymerase to promoter sequences. The major σ subunit of *E. coli*, σ^{70}, is responsible for RNA polymerase binding to the basic promoter motif (TATAAT at −10 and TTGACA at −35). Direct biochemical purification of RNA polymerase from many bacterial species for use in in vitro transcription systems has been relatively straightforward, owing in part to the ability to grow large quantities of these microorganisms. Such an approach is not practical for chlamydiae; the poor growth of this bacterium in culture makes it exceedingly difficult to generate the necessary starting material for such large-scale purifications. Instead, it is likely that techniques developed for the isolation of rare protein species, such as immunoadsorption to antibody columns, may be necessary for chlamydial RNA polymerase characterization. To this end, we have turned our efforts towards cloning and overexpressing the subunits of chlamydial RNA polymerase in *E. coli* to facilitate its further purification for subsequent use in vitro in the analysis of promoter structure and of protein factors important in the control of chlamydial gene expression.

Using a strategy that makes use of the polymerase chain reaction (PCR) to directly amplify related sequences from the chlamydial genome, we cloned and characterized a chlamydial homolog of σ^{70} (8). After identifying regions of the α, β, and β' subunits of *E. coli* RNA polymerase conserved in other organisms, we have now extended this PCR-based approach to the cloning of the β and β' subunits of RNA polymerase from a murine strain of *C. trachomatis*.

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MATERIALS AND METHODS

Reagents. Products were obtained from the following sources and were used according to the manufacturer's specifications: restriction enzymes, bacterial alkaline phosphatase, and T4 DNA ligase, New England BioLabs, Inc. (Beverly, Mass.); T4 polynucleotide kinase, Boehringer Mannheim Biochemicals (Indianapolis, Ind.); DNA polymerase I, Pharmacia Fine Chemicals (Piscataway, N.J.); ^{32}P-containing radioisotopes, Amersham Corp. (Arlington Heights, Ill.); [^{35}S]methionine, ICN (Irvine, Calif.); *Thermus aquaticus* DNA polymerase, Cetus Corp (Emeryville, Calif.); SeaPlaque and Seakem agarose, FMC Bioproducts (Rockland, Maine); ampicillin, kanamycin, rifampin, protein A-Sepharose CL-4B, and DNase I, Sigma Chemical Co. (St. Louis, Mo.); dimethyl-3,3'-dithiobis-propionimidate (DTBP), Pierce Chemical Co. (Rockford, Ill.); and protein molecular weight markers, Bethesda Research Laboratories (Bethesda, Md.).

Nucleic acid preparation and analysis. Chlamydial DNA from the mouse pneumonitis (MoPn) strain of *C. trachomatis* was prepared as described previously (9). Human DNA was isolated from HeLa cells grown in culture (16). *E. coli* DNA was prepared from strain TG1 (T. J. Gibson, Ph.D. thesis, Cambridge University, Cambridge, England) as described before (16). Standard recombinant DNA methods were used for nucleic acid preparation and analysis (16). Restriction fragments were subcloned into a pGEM7Zf (Promega, Madison, Wis.) plasmid vector. Southern blotting was carried out as described previously (9). Radioactive DNA probes were labeled by nick translation or by 5'-end labeling with T4 polynucleotide kinase (16).

Synthetic oligonucleotides. The following single-stranded oligonucleotide primers were synthesized by the Biomedical Resource Center at the University of California, San Francisco: α5' primer, CCGAATTCCA(TC)GA(AG)TA(TC)T C(AGTC)AC; α3' primer, GGCTCGAG(AGT)AT(AG) ATC(AGCT)GC(AGCT)GC(AGCT)G; β5' primer, CC GAATTCAA(TC)ATGCA(AG)CG(ATGC)CA; β3' primer, GGCTCGAG(AG)TC(TC)TC(AG)AA(AG)TT(AG)T A; β' 5' primer, CCGAATTCAT(TAC)CA(AG)GC(A GCT)TT and CCGAATTCGG(AGCT)AA(AG)CG(ACG T)GT(AGCT)GA;β'3' primer, GGCTCGAG(AG)TC(A T)AA(AG)TC(AGCT)GC(AG)TT and GGCTCGAG(A G)AAG(ACGT)GC(TC)TG(ATG)AT(AGCT)CC.

PCR. The PCR was performed with a Cetus/Perkin-Elmer DNA thermocycler. Reaction mixtures (100 μl) contained 100 pmol of the 5' and 3' primers, all four dNTPs at 1 mM each, 50 mM KCl, 10 mM Tris chloride (pH 8.0), 2 mM MgCl$_2$, 0.01% gelatin, 1 μg of DNA, and 2.5 U of *T. aquaticus* DNA polymerase. The reaction mixture was overlaid with a drop of paraffin oil and subjected to 35 cycles consisting of a 2-min denaturation period at 94°C, a 2-min annealing period at 37°C, and a 2-min extension period at 72°C. After analysis of the PCR product on a 1.5% low-melting-point agarose gel (SeaPlaque), the amplification product was purified from the gel, followed by isolation with glass beads (GeneClean; Bio101, La Jolla, Calif.). The gel-purified product was digested with *Eco*RI and *Xho*I and cloned into pGEM7Zf previously digested with *Eco*RI and *Xho*I, followed by treatment with bacterial alkaline phosphatase.

Preparation and screening of a chlamydial DNA library. Chlamydial DNA was digested with *Eco*RI and cloned into a pUC8 (Pharmacia, Piscataway, N.J.) vector previously cleaved with *Eco*RI and dephosphorylated with bacterial

alkaline phosphate. A total of 900 colonies were stabbed onto L-broth plates containing ampicillin (50 μg/ml). After overnight growth at 37°C, the plates were overlaid with Hybond filters (Amersham Corp., Arlington Heights, Ill). Filters bearing colonies were soaked in 0.5 M NaOH–1.5 M NaCl, followed by 0.5 M Tris chloride (pH 8.0)–1.5 M NaCl. Following UV light cross-linking, the filters were hybridized as described previously (9) to a 5'-end-labeled probe made from the PCR product. The *Eco*RI fragments from the clones that hybridized to this probe were then recloned into the *Eco*RI site of pGEM7Zf for further analysis.

DNA sequencing. The dideoxy chain termination method of DNA sequencing (23) was carried out on double-stranded fragments cloned into pGEM7Zf with the Sequenase kit (United States Biochemical Corp., Cleveland, Ohio). Sequencing reactions were primed with oligonucleotides homologous to the T7 and SP6 promoters (Promega Biotech, Madison, Wis.) flanking the cloned inserts in the pGEM7Zf vector. Most of the sequencing was carried out on one strand of the duplex only.

Overproduction of bacterially encoded proteins. Overproduction of proteins encoded by cloned genes of interest was accomplished by introducing the corresponding plasmid into a strain of *E. coli* harboring pGP1-2 [HMS262(pGP1-2)] (32), a pGEM-compatible plasmid that encodes the phage T7 RNA polymerase under control of a thermolabile lambda repressor. The following plasmids were constructed in pGEM7Zf (see Fig. 4B): pBETA (L7/L12, full-length β gene, and the 5' half of β'), p291 (the 5' *Eco*RI-*Cla*I fragment from pBETA), and p280 (5' *Eco*RI-*Nsi*I fragment from pBETA). Qualitative induction of the plasmid-encoded gene product was carried out as follows. Strains were grown at 30°C in L-broth (16) containing kanamycin (50 μg/ml) and ampicillin (100 μg/ml) to an A_{600} of 0.6. Samples (1 ml) were pelleted and suspended in M9 minimal medium (16) containing thiamine and all amino acids except methionine. The cultures were then transferred to 42°C for 20 min, at which time rifampin (100 μg/ml) was added. Following further incubation at 42°C for 10 min, [^{35}S]methionine was added (50 μCi/ml), and the cultures were grown at 37°C for 30 min. The bacteria were pelleted and lysed in 100 μl of Laemmli buffer (12) containing 5% (vol/vol) β-mercaptoethanol. Polyacrylamide gel electrophoresis (PAGE) of the protein products was carried out on 12% sodium dodecyl sulfate (SDS)-polyacrylamide slab gels (12), as modified (2).

Quantitative overproduction of the plasmid-encoded gene product was carried out similarly with the following modifications. One liter of HMS262(pGP1-2, p291) bacteria was grown and thermoinduced for protein expression in L-broth as described above. The bacterial cells were pelleted and frozen. After thawing, the bacteria were lysed by treatment with 110 mg of lysozyme in TEN buffer (10 mM Tris [pH 7.5], 1 mM EDTA, 0.1 M NaCl) on ice for 15 min, followed by the addition of Nonidet P-40 to 0.2% for 10 min. Then, 2 mg of DNase I was added, and the sample was stirred on ice for 1 h, followed by shearing of the remaining undigested DNA with a Polytron homogenizer/sonicator (model Ls10-35; Kinematica, Lucern, Switzerland) for 30 s. The sample was then centrifuged at 10,000 × g for 10 min, and the pellet was suspended by boiling in 3 ml of Laemmli buffer (12) containing 5% (vol/vol) β-mercaptoethanol for 20 min. The material was electrophoresed on a preparative 12% SDS–polyacrylamide gel (12). A strip from the gel was stained briefly in 10% acetic acid containing 0.25% Coomassie blue R250, and the band corresponding to the overexpressed β protein fragment was excised. This excised gel fragment was

immersed in 5 volumes of Laemmli electrophoresis buffer (12) and broken into fragments by treatment with the Polytron homogenizer/sonicator for 30 s. β-Mercaptoethanol was added to 0.1%, and the protein was eluted from the gel by agitation at room temperature for 12 to 18 h. The acrylamide was pelleted by centrifugation, and the protein in the supernatant was precipitated by the addition of 3 volumes of methanol, followed by incubation on ice for 2 h. The precipitate was collected by centrifugation and suspended in phosphate-buffered saline (PBS) containing 0.1% SDS. A 150-μg amount of the gel-purified protein was injected into a rabbit, followed by boosting. The antibody production was carried out by Caltag Corp. (Berkeley, Calif.).

In vivo labeling of chlamydial proteins. Chlamydia-infected HeLa cells were incubated in Dulbecco modified medium lacking methionine and cysteine in the presence of cycloheximide (50 μg/ml) for 30 min, followed by pulse labeling with [^{35}S]methionine (100 μCi/ml) for 30 min at various times during infection. After extraction with lysis buffer (10% glycerol, 50 mM Tris chloride [pH 7.5], 150 mM NaCl, 0.2% Triton X-100, 1 μg of aprotinin [Sigma Chemical Co., St. Louis, Mo.] per ml, 1 mM phenylmethylsulfonyl fluoride [Sigma], and 1 mM leupeptin [Sigma], samples were electrophoresed on 10% SDS–polyacrylamide gels.

Immunoblot analysis and immunoprecipitations. Immunoblots were carried out as described before (33), with 2% gelatin as a blocking agent. Preimmune or immune serum was used at a dilution of 1:200. Alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (Promega) was used at a dilution of 1:7,500 for the second antibody reaction. To compete out antibodies to *E. coli* present in the rabbit antiserum, an *E. coli* protein lysate (Promega) was incubated at a concentration of 200 μg/ml with a 1:800 dilution of the antiserum prior to incubation with the Western immunoblot of pBETA and its truncated derivatives expressed in *E. coli*.

Immunoprecipitations were performed as described previously (10) with the following modifications. Dishes (100 mm) or T75 flasks of subconfluent HeLa cells (uninfected or infected with chlamydiae for 12 h) were labeled with [^{35}S]methionine (500 μCi/ml) for an additional 8 h in methionine- and cysteine-free Dulbecco modified medium containing cycloheximide (50 μg/ml). Cells were removed from the dishes by gentle agitation with 2 ml of lysis buffer, followed by a brief centrifugation of the supernatant at 12,000 × *g*. The supernatant was added to 20 to 50 μl of protein A-Sepharose CL-4B beads along with preimmune or immune antiserum (5 μl of serum per 10 μl of beads) and rocked for 12 to 18 h at 4°C. The protein A-Sepharose beads were washed with RIPA buffer (50 mM Tris chloride [pH 7.5], 0.5 M NaCl, 20 mM EDTA, 0.2% Triton X-100, 0.05% SDS, 1% deoxycholate), followed by washes with PBS. The bound antigen was eluted in Laemmli sample buffer (12) containing 5% (vol/vol) β-mercaptoethanol and electrophoresed on 10% SDS–polyacrylamide slab gels. The radiolabeled product was visualized by fluorography.

For the in vivo cross-linking experiments, chlamydial infections were carried out as described above. The medium was removed, and 2 ml of DTBP (5 mg/ml in PBS) was added to the plates. Following gentle agitation at room temperature for 30 min, the DTBP-PBS was removed and the plates were washed three times with PBS. Immunoprecipitations were then carried out as above. For the in vitro cross-linking experiments, the cell lysates were adsorbed overnight to protein A-Sepharose-antibody. Then, 1/10 volume of DTBP (5 mg/ml in PBS) was added to the lysates, and the samples

were incubated by rocking at room temperature for 30 min. The immunoprecipitates were then washed as described above. Chlamydia-infected cells were exposed to a heat shock stress by incubating the dishes in a 45°C water bath for 10 min prior to cross-linking or extraction in lysis buffer. We have shown that these conditions induce a heat shock response in *C. trachomatis* (J. Engel, J. Pollack, E. Perara, and D. Ganem, unpublished data).

RESULTS

Cloning of the β subunit of chlamydial RNA polymerase. The Dayhoff protein data base was searched for proteins with homology to the subunits of *E. coli* core RNA polymerase (6, 19–21). Significant homologies to the *E. coli* RNA polymerase α chain were found in the RNA polymerases of vaccinia virus (7), *B. subtilis* (5), liverwort (18), and common tobacco plant chloroplast (28); significant homologies to the *E. coli* RNA polymerase β chain were found in the chloroplast RNA polymerase of the liverwort (18) and the common tobacco plant (17); and significant homologies to the *E. coli* RNA polymerase β' chain were found in the chloroplast RNA polymerase of liverwort (18), RNA polymerase II of *Drosophila melanogaster* (4) and *Saccharomyces cerevisiae* (1), and the vaccinia virus RNA polymerase (7) (Fig. 1).

Reasoning that regions of the protein conserved between enterobacteria, plant chloroplast, and eucaryotic RNA polymerase would probably be conserved in the RNA polymerase subunit homologs of other gram-negative organisms, we synthesized degenerate oligonucleotides from these regions to use as primers (Fig. 1 and Materials and Methods) in a PCR reaction to amplify the corresponding region from chlamydiae. With the β subunit primers, a discrete PCR product was generated in a reaction in which the MoPn strain of chlamydial DNA was the template and was identical in size to the corresponding fragment generated by a PCR in which *E. coli* DNA was the template (data not shown); however, no reaction products were seen when the α or β' primer was used in a PCR with MoPn DNA as the template (data not shown).

The PCR product directed by the β primers was 5'-end labeled with T4 polynucleotide kinase in the presence of [γ-^{32}P]ATP and was used as a probe to screen a plasmid library of chlamydial *Eco*RI fragments of MoPn DNA cloned into the vector pUC8. Ten positive clones were found after screening 900 colonies; restriction digest analysis demonstrated that all 10 clones contained the same 6.5-kilobase (kb) *Eco*RI fragment insert. The PCR fragment was cloned into pGEM7Zf (see Materials and Methods), radiolabeled by nick translation, and used to probe Southern blots of MoPn genomic DNA cleaved with *Eco*RI, *Bam*HI, or *Hin*dIII. A single band of hybridization was observed to chlamydial DNA cut with each of these enzymes, suggesting that the sequences detected are present in a single copy of the chlamydial genome (Fig. 2, lanes 1 to 3). The cloned 6.5-kb *Eco*RI fragment hybridized to the PCR-generated probe (lane 6) and comigrated with the genomic *Eco*RI fragment (lane 1) that hybridized to the PCR probe. The PCR-generated β probe did not cross-hybridize to *E. coli* DNA (lane 4) or human DNA (lane 5) under stringent hybridization and wash conditions. The latter control was carried out to verify that the cloned PCR product was indeed of chlamydial origin, as all preparations of chlamydial DNA are contaminated with host cell (in this case, HeLa) DNA.

Evidence that the 6.5-kb *Eco*RI fragment encodes the chlamydial homolog of the β subunit gene. Limited DNA se-

	67	110
E. coli a subunit	HEYST	VTAaDI
	63	107
B. subtilis a subunit	HEfST	VTAaDI
	72	116
Liverwort a subunit	HEYST	iTAqDI
	72	116
Tobacco a subunit	HEYST	VTAqDI
	469	513
Vaccinia virus	fEYra	qkmfsn
PCR PROBE	HEYST	VTAA/QDI
	5'	3'

	684	809
E. coli ß subunit	NMQRQ	GYNfED
	542	657
Liverwort ß subunit	NMQRQ	GYNfED
	547	662
Tobacco ß subunit	NMQRQ	GYNsED
PCR PROBE	NMQRQ	GYNFED
	5'	3'

	345	433	458
E. coli ß' subunit	GKRVD	GIQAF	NADFD
	373	452	487
Liverwort ß' subunit	GKRVD	GIQAF	NADFD
	241		
D. melanogaster PolII	GKRVD		
			480
S. cerevisiae PolII			NADFD
			413
Vaccinia Pol			NADFD
PCR PROBE	GKRVD	GIQAF	NADFD
	5'	5'/3'	3'

FIG. 1. Conserved regions in the core polymerase α, β, and β' subunit proteins of E. coli. The α subunit of E. coli (21) is compared with the B. subtilis α subunit (5), the liverwort (Marchantia polymorphia) chloroplast α subunit (18), the common tobacco plant chloroplast α subunit (28), and the vaccinia virus RNA polymerase (7). The β subunit of E. coli (6, 19) is compared with the liverwort chloroplast β subunit (18) and the common tobacco plant chloroplast β subunit (17). The β' subunit of E. coli (20) is compared with the liverwort chloroplast β' subunit (18), RNA polymerase II (PolII) from D. melanogaster (4) and S. cerevisiae (1), and the vaccinia virus RNA polymerase (Pol) (7). Amino acids conserved among all or most of the species compared are denoted by capital letters, and the PCR probes used in the studies are underlined. The degenerate oligonucleotide primer derived from the peptide sequence GIQAF was used as both a 5' and a 3' primer in PCRs attempting to amplify the chlamydial β' gene. The numbers refer to the amino acid position in the protein.

quencing was carried out on portions of this 6.5-kb EcoRI fragment to verify that it encoded the β subunit gene homolog of C. trachomatis. Figure 3A illustrates the sequencing strategy and demonstrates that regions of this chlamydial DNA fragment encoded a protein with homology to the E. coli β subunit of RNA polymerase (amino acids 644 to 812, Fig. 3B). There was also significant homology to the plant chloroplast RNA polymerase β subunit (data not shown). Notably, the amino acid sequence predicted from the DNA sequence upstream of the region from which the 5' primer was chosen also conserved the homology observed between the E. coli and plant chloroplast β subunit proteins.
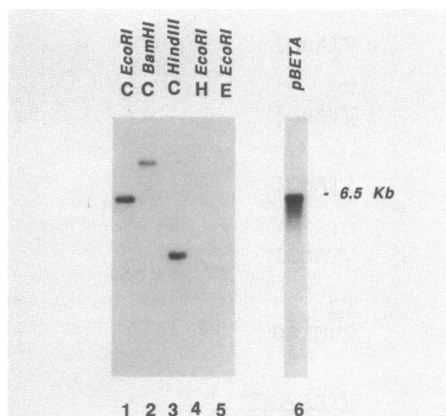
FIG. 2. Southern blot analysis of chlamydial DNA and the cloned putative β subunit fragment probed with the PCR products. MoPn DNA (1 μg) (lanes 1 to 3), 5 μg of HeLa cell DNA (lane 4), 1 μg of *E. coli* DNA (lane 5), or 100 ng of pBETA (lane 6) was cleaved by *Eco*RI (lanes 1, 4, 5, and 6), BamHI, or *Hin*dIII, electrophoresed on a 1% agarose gel, and transferred to a nylon filter. Lanes 1 to 5 were hybridized to 10^6 cpm of a ^{32}P-labeled DNA probe made by nick translation of pBETA. Lane 6 was hybridized to 10^6 cpm of a probe made by 5'-end labeling of the PCR products by T4 polynucleotide kinase in the presence of [γ-^{32}P]ATP. Southern blot analysis was carried out as described previously (9).

Sequencing in from the *Cla*I site (Fig. 3A) revealed an additional region of amino acid homology to the *E. coli* and plant chloroplast proteins (amino acids 442 to 568, Fig. 3B). Together, these results leave little doubt that the cloned sequences indeed represent the chlamydial β subunit.

Sequencing around other restriction enzyme sites in the 6.5-kb *Eco*RI fragment generated two additional observations. First, upstream of the β subunit gene were sequences coding for a protein homologous to the eubacterial ribosomal L7/L12 protein (Fig. 3C). The *C. trachomatis* homolog was 41% identical to the *E. coli* protein; an additional 43% of the residues were conserved. Interestingly, the chlamydial L7/L12 protein was even more closely related to the cognate protein in the bacterium *Desulfovibrio vulgaris*. These two proteins were 51% identical over the 122 amino acid residues (data not shown). Second, downstream of the β subunit gene was found the gene encoding the β' subunit homolog. The amino acid sequence surrounding the *Xba*I site showed 48% identity over a 116-amino-acid stretch with the *E. coli* β' subunit, strongly suggesting that this gene does encode the β' subunit of *C. trachomatis* RNA polymerase (Fig. 3D). This gene order, L7/L12-β-β', is reminiscent of the *rpoBC* operon of *E. coli* (21), which sequentially encodes L11, L1, L10, L7/L12, β, and β' in the 5' to 3' direction. Though we have not determined whether L11, L1, and L10 are encoded on DNA that is adjacent to the 5' end of the chlamydial 6.5-kb *Eco*RI fragment, our findings indicate that part, if not all, of the structure of this operon is conserved in chlamydiae.

β subunit gene of chlamydial RNA polymerase encodes a 150-kDa protein. The 6.5-kb *Eco*RI fragment and the 5' *Eco*RI-*Nsi*I and 5' *Eco*RI-*Cla*I fragments derived from this *Eco*RI fragment were subcloned into the vector pGEM7Zf so that the coding regions of these genes lay downstream of the bacteriophage T7 promoter (plasmids pBETA, p280, and p291, respectively; Fig. 4C). These plasmids were transformed into a strain of *E. coli* containing a pGEM-compatible plasmid, pGP1-2, that expresses the T7 polymerase gene

under control of a lambda promoter and a thermolabile lambda repressor (*c*I857) (32). Upon thermoinduction of strain HMS262(pGP1-2, pBETA), pBETA directed the synthesis of an approximately 150-kilodalton (kDa) (Fig. 4A, lane 3) not seen in the control strain containing pGEM7Zf [HMS262(pGP1-2, pGEM7Zf)] without an insert (lane 6). This presumptive β protein comigrated with the lower band of a protein doublet seen in [^{35}S]methionine-pulse-labeled chlamydia-infected cells (lane 2); this doublet had the mobility characteristic of β (lower band) and β' (upper band) proteins observed in eubacteria. In some experiments (though not in the gel chosen for Fig. 4), HMS262(pGP1-2, pBETA) was observed to direct the synthesis of an additional protein product of approximately 40 kDa which most likely represents the truncated β' protein product that pBETA is predicted to encode. Strains bearing plasmids p280 and p291, which contain C-terminal deletions of the β gene, directed the synthesis of protein products of appropriately sized truncated polypeptides (ca. 80 and 42 kDa, respectively; lanes 4 and 5). The 40-kDa protein product encoded by HMS262(pGP1-2, pBETA) was not present in the radiolabeled protein products synthesized by strains bearing p280 and p291 (data not shown), corroborating the identification of the 40-kDa protein as the truncation product directed by the N-terminal fragment of the β' gene present in pBETA.

Generation and characterization of a rabbit polyclonal antiserum raised to the chlamydial β subunit protein. Strains HMS262(pGP1-2, p280) and especially HMS262(pGP1-2, p291) reproducibly yielded larger amounts of the newly synthesized β subunit protein fragment than did HMS262 (pGP1-2, pBETA) (which encodes the full-length β polypeptide). We chose to purify the polypeptide encoded by p291 in strain HMS262(pGP1-2) for injection into rabbits. Liter quantities of the strain HMS262(pGP1-2, p291) were thermoinduced for expression, and the detergent-insoluble proteins from these cells were electrophoresed on SDS-PAGE gels (see Materials and Methods). The band corresponding to the thermoinduced β polypeptide fragment was excised from the gel and eluted for injection into rabbits. A polyclonal antiserum was obtained after several boostings that specifically recognized the appropriately sized β subunit fragments on immunoblots of *E. coli* extracts from strain HMS262 carrying pGP1-2 plus the p280 or p291 plasmid (Fig. 4B, lanes 2 and 3). On this immunoblot, the antiserum did not recognize the full-length chlamydial β protein synthesized by pBETA; the likeliest explanation for this observation is that synthesis of the full-length β protein by pBETA is very inefficient compared with that of the truncated products produced by p291 and p280 and may have been below the detection limit of this immunoblot.

Immunoprecipitation of RNA polymerase from chlamydia-infected cells by the β antibody. The antibody to the β subunit was produced to assist in the purification of RNA polymerase from chlamydiae; we therefore asked whether the β antibody could selectively immunoprecipitate the entire multisubunit enzyme. Cell lysis conditions were selected to minimize the amount of detergent in an effort to prevent dissociation or denaturation of the multisubunit enzyme (10% glycerol and 0.2% Triton X-100; see Materials and Methods). Figure 5 illustrates an immunoprecipitation of extracts prepared from cells infected with chlamydiae for 8 to 20 h, labeled with [^{35}S]methionine, and electrophoresed on 10% SDS-PAGE gels. Lanes 15 and 18 illustrate that immune serum selectively immunoprecipitated a 150-kDa protein that comigrated with the lower band of the charac-

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A        EcoRI                    ClaI     NsiI              XbaI    EcoRI

pBETA   ┌──[////]──████████████████████████████──[//////////]──┐
        L7/L12                          ß                    ß'
        ──────►              ──►  ◄──┼──►              ──►
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                 450       460       470       480       490       500
B  C. trach ß  IDDIDHLANRRVRSVGELIQNHSRSGLARMEKIVRERMNLFDFSSDTLTPGKIISAKGLVS
               .:::::::.:::.:::::. .:. : ::.:.:. :.::..: :. ::: : ..:.:: . .
   E. coli ß  VDDIDHLGNRRIRSVGEMAENQFRVGLVRVERAVKERLSLGDL--DTLMPQDMINAKPISA


                 510       520       530       540       550       560
   C. trach ß  VLKDFFSRSQLSQFMDQTNPVAELTHKRRLSALGPGGGLNRERAGFEVRDVHSSHYGRICPIETPEGPK
               ..:.::..:::::::: :.::..:.:::::.:::::::::.:::::::::::::..::::.:::::::::.
   E. coli ß  AVKEFFGSSQLSQFMVQNNPLSEITHKRRISALGPGGGLTRERAGFEVRDVHPTHYGRVCPIETPEGPN


                 650       660       670       680       690       700
   C. trach ß  LTDQSTVTHMDVSPKQLVSVVTGLIPFLEHDDANRALMGSNMQRQAVPLLKTEAAIV
               : ... :..:::::.:.::: ..::::::::::::::::.:::::::: :... ..:
   E. coli ß  LFSRDQVDYMDVSTQQVVSVGASLIPFLEHDDANRALMGA𝗡𝗠𝗤𝗥𝗤AVPTLRADKPLV
                                                         5'


                 710       720       730       740       750       760
   C. trach ß  GTGLEGRAAKDSGAIVVAQEDGVVEYVDSYEIVV-----AKKNNPTLKDTYPLKKFLRSNSGTMH
               :::.:  .: :::..::. .:::.:::. ::.      .. . . :.: :.:. ::: .:
   E. coli ß  GTGMERAVAVDSGVTAVAKRGGVVQYVDASRIVIKVNEDEMYPGEAGIDIYNLTKYTRSNQNTCI


                 780       790       800       810
   C. trach ß  GDVLADGPATDKGELALGKNVLVAFMPQYGYNFED
               :::::::::.:: :::::::.:. ::::: :::::::
   E. coli ß  GDVLADGPSTDLGELALGQNMRVAFMPWN𝗚𝗬𝗡𝗙𝗘𝗗
                                            3'
```

```
                 10        20        30        40        50        60
C  C. trach.L7/L12  TESLETLVEQLSRLTVLELSQLKKMLEEKWDVTAAAPVVAVAGAAAAGDRPASAEPTEFA
                    . ..:.... ..:.... .: . .:::..:.:::.:...:...:..   : :::.
   E. coli L7/L12   SITKDQIIEAVAAMSVMDVVELISAMEEKFGVSAAAAVAVAAGPVEAAE-----EKTEFD
                    10        20        30        40        50


                 70        80        90        100       110
   C. trach. L7/L12  VILEDVPADKKIGVLKVVREVTGLALKEAKEMTEGLPKTVKEKTSKSDAEDTVKKLQEAG
                     :::  .......:.:.::..::..::::::::::..: :  ..:: .::.::: : :.:::
   E. coli L7/L12    VIL-KAAGANKVAVIKAVRGATGLGLKEAKDLVESAPAALKEGVSKDDAEALKKALEEAG
                     60        70        80        90        100       110


                 120
   C. trach.L7/L12  AKAVAK
                    :... .:
   E. coli L7/L12   AEVEVK
                    120
```

```
D  C. trach ß'  SRIGNEKGMTASDLERVIYYEEYVVIDPGNTDLVKKQLLNDAKYREVVEKWGKDAFVAK
                 ::::   .:. .:.:::.:.:.::::. : :.: ..:.:..: .......: :.: ::
   E. coli ß'   PSRIGLLLDMPLRDIERVLYFESYVVIEGGMTNLERQQILTEEQYLDALEEFG-DEFDAK
                 130       140       150       160       170


   C. trach ß'  MGGEAVYDLLKSEDLESLLGELKDRLRKTKSQQARMKLAKRLKIVEGFVSSSNRPEW
                ::.::. .::::: :::  ....: :...:. :.::.::.:...:.:: :.:.:::
   E. coli ß'  MGAEAIQALLKSMDLEQECEQLREELNETNSETKRKKLTKRIKLLEAFVQSGNKPEW
                180       190       200       210       220       230
```
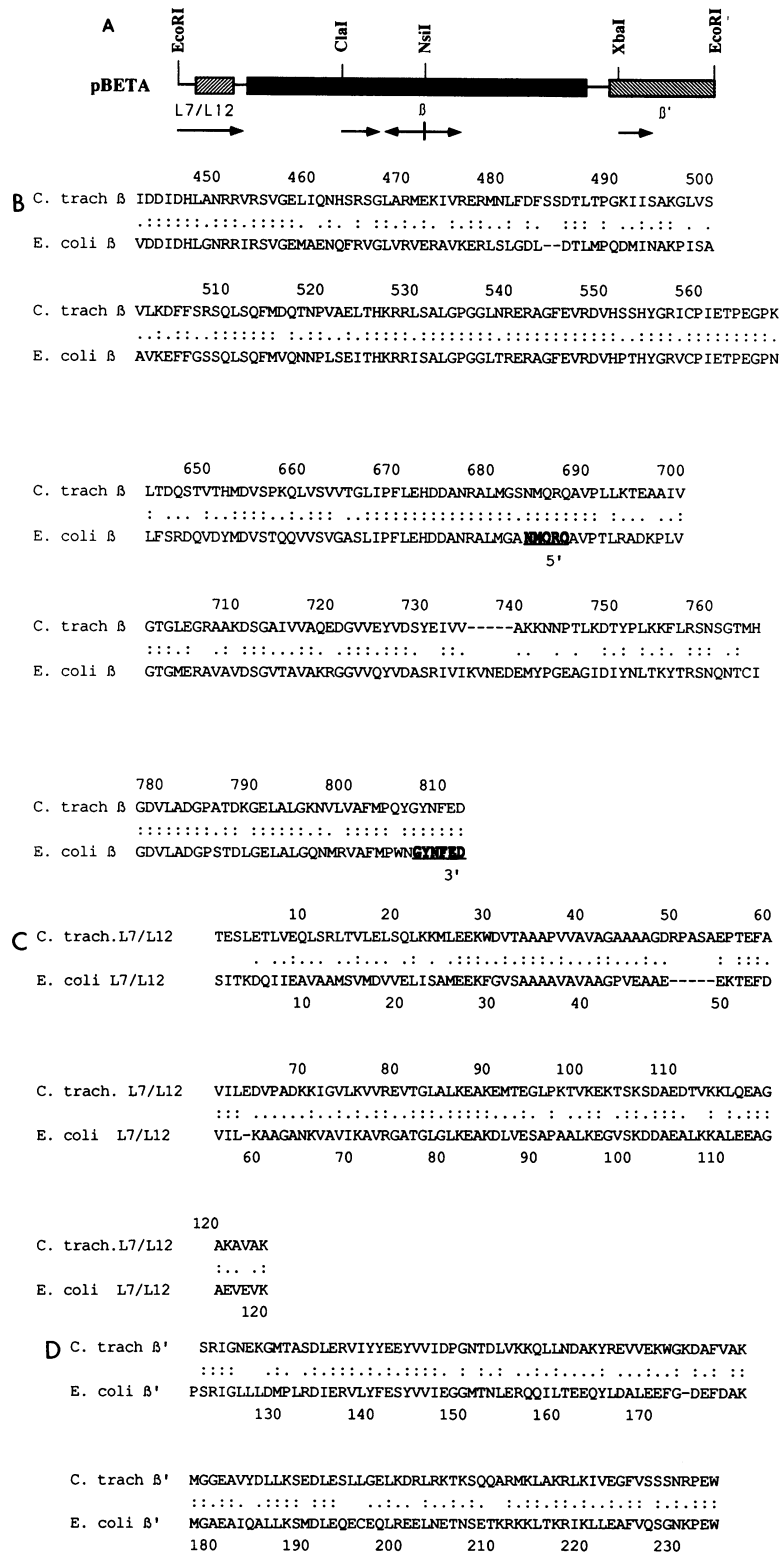
FIG. 3. (A) Organization of the pBETA clone and sequencing strategy. Arrows indicate the regions of pBETA whose sequence was determined by the chain termination method (23). Selected regions of the predicted amino acid sequence of the *C. trachomatis* (C. trach.) β (B), L7/L12 (C), and β' (D) proteins are shown and are compared with the corresponding region of the cognate *E. coli* protein. Identical amino acids are designated by two dots, and conserved amino acids are indicated by a single dot. The portion of the β sequence from which the β PCR primers were derived is underlined and highlighted. Note that the derived amino acid sequence for the *C. trachomatis* β protein, shown in the upper portion of panel B, corresponding to the *E. coli* β subunit residues 644 to 812, is missing the amino acid residues between 766 and 777.
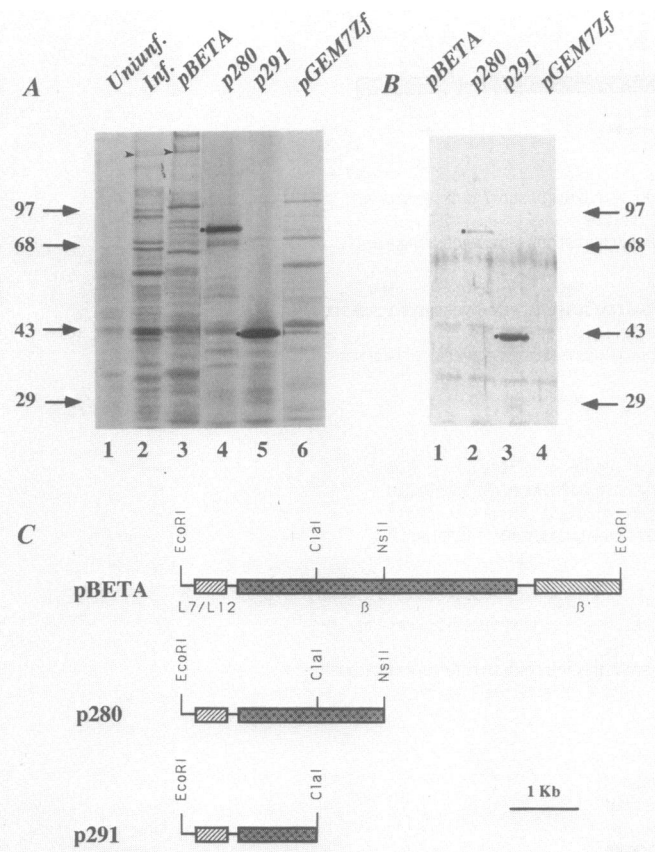
FIG. 4. Overexpression of the *C. trachomatis* β protein in *E. coli*. (A) Autoradiograph of the SDS-PAGE gel of plasmid-encoded gene products that were thermoinduced for expression in a strain of *E. coli* containing the T7 polymerase gene on a plasmid (pGP1-2) (32) plus pBETA (lane 3), p280 (lane 4), p291 (lane 5), or pGEM7Zf (lane 6), as described in Materials and Methods. Lanes 1 and 2 show the protein products labeled with [35S]methionine (500 μCi/ml) in the presence of cycloheximide (50 μg/ml) from uninfected HeLa cells (lane 1) or chlamydia-infected HeLa cells at 18 h.p.i. (lane 2). The full-length β polypeptide is indicated by the arrow in lanes 2 and 3. Although on this autoradiograph the β polypeptide expressed in strain HMS262(pGP1-2, pBETA) appears to comigrate with the upper band of the doublet, on SDS-PAGE gels that better resolved the doublet, it clearly migrated with the lower band. The truncated β polypeptides encoded by plasmids p280 and p291 are indicated by a dot. Sizes are shown in kilodaltons. (B) Western blot of a gel similar to that shown in panel A and immunoblotted to the β antiserum. The β antiserum was incubated with *E. coli* lysate (200 μg/ml) for 30 min prior to binding to the immunoblot. Plasmids: pBETA (lane 1), p280 (lane 2), p291 (lane 3), and pGEM7Zf (lane 4). The truncated protein products specifically recognized by the antiserum are shown by a dot. (C) Structure and relevant restriction sites in the clones used for expression of the β subunit in *E. coli*. The shaded regions represents the coding regions of the L7/L12, β, and β' genes, as marked. The construction of the clones is described in Materials and Methods.

teristic 150-kDa doublet (lane 13); preimmune serum did not immunoprecipitate the 150-kDa polypeptide (lane 14). Likewise, no radiolabeled protein products from uninfected HeLa cells were immunoprecipitated by the immune or preimmune serum (lanes 11 and 12). Several other bands were visible in the immunoprecipitate of chlamydia-infected cells (lanes 15 and 18); they may represent some of the other subunits of RNA polymerase (α or σ), degradation products
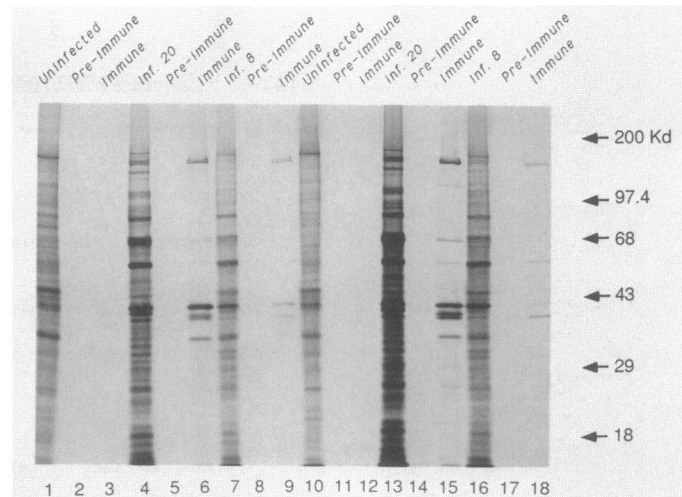

FIG. 5. Immunoprecipitation of chlamydia-infected HeLa cell extracts with the β antibody in the presence or absence of cross-linking. In lanes 1 to 9, the cells were briefly exposed to the reversible chemical cross-linker DPBT, as described in Materials and Methods. Lane 1, Lysate from [35S]methionine-labeled uninfected HeLa cells. Lane 2, Immunoprecipitation of [35S]methionine-labeled uninfected HeLa cells with preimmune serum. Lane 3, Immunoprecipitation of [35S]methionine-labeled uninfected HeLa cells with immune serum. Lane 4, Lysate from infected HeLa cells pulse-labeled from 12 to 20 h.p.i. Lane 5, Immunoprecipitation with preimmune serum of infected HeLa cells pulse-labeled from 12 to 20 h.p.i. Lane 6, Immunoprecipitation with immune serum of infected HeLa cells pulse-labeled from 12 to 20 h.p.i. Lane 7, Lysate from infected HeLa cells pulse-labeled from 4 to 8 h.p.i. Lane 8, Immunoprecipitation with preimmune serum of HeLa cells pulse-labeled from 4 to 8 h.p.i. Lane 9, Immunoprecipitation with immune serum of HeLa cells pulse-labeled from 4 to 8 h.p.i. Lanes 10 to 18, No cross-linking prior to immunoprecipitation. Lane 10, Lysate from [35S]methionine-labeled uninfected HeLa cells. Lane 11, Immunoprecipitation of [35S]methionine-labeled uninfected HeLa cells with preimmune serum. Lane 12, Immunoprecipitation of [35S]methionine-labeled uninfected HeLa cells with immune serum. Lane 13, Lysate from infected HeLa cells pulse-labeled from 12 to 20 h.p.i. Lane 14, Immunoprecipitation with preimmune serum of infected HeLa cells pulse-labeled from 12 to 20 h.p.i. Lane 15, Immunoprecipitation with immune serum of infected HeLa cells pulse-labeled from 12 to 20 h.p.i. Lane 16, Lysate from infected HeLa cells pulse-labeled from 4 to 8 h.p.i. Lane 17, Immunoprecipitation with preimmune serum of HeLa cells pulse-labeled from 4 to 8 h.p.i. Lane 18, Immunoprecipitation with immune serum of HeLa cells pulse-labeled from 4 to 8 h.p.i. Following immunoprecipitation, the specifically bound proteins were eluted from the protein A-Sepharose by boiling in 40 μl of 2× Laemmli buffer (12). The entire sample was loaded on a 10% SDS–polyacrylamide gel and fluorographed. Lanes 1, 4, and 7 represent 1/50 of the cell lysate precipitated in the remaining lanes. Protein molecular mass standards are indicated on the right.

of the β subunit, or cross-reactivity with other chlamydial proteins. The first possibility is unlikely, as this immune serum did not reproducibly precipitate the β' subunit of RNA polymerase. The other two hypotheses have not been evaluated further.

We next asked whether the polymerase holoenzyme could be immunoprecipitated by the β antiserum if the infected cells were exposed first to a cross-linking agent. While this approach would not be useful for the purification of chlamydial RNA polymerase, it could potentially identify the α and σ subunits of this enzyme. Chlamydia-infected HeLa cells were labeled with [35S]methionine and then briefly

exposed to the cleavable cross-linker DTBP. Lysates of cells infected for 8 or 20 h were immunoprecipitated with preimmune or β antiserum and compared with lysates of cells that had undergone the same treatment except that the cross-linking step had been omitted. An additional labeled protein band was detectable in the immunoprecipitates of cross-linked lysates from chlamydia-infected cells (Fig. 5, lanes 9 and 6) that was absent from uninfected cells (Fig. 5, lane 3). This band comigrated with the upper band of the characteristic β-β' doublet and presumably represents the β' subunit of chlamydial RNA polymerase. No new labeled protein bands of the size expected for σ (68 kDa [8]) or α (ca. 40 kDa by analogy to other procaryotes) could be detected specifically in any of the immunoprecipitates exposed to the cross-linker DTBP (data not shown); thus, except for the presence of β', there were no other differences in the [35S]methionine-labeled proteins immunoprecipitated in the cross-linked compared with the non-cross-linked samples. This analysis was complicated by the fact that several minor protein species other than the β polypeptide were present in the immunoprecipitates that had not been cross-linked (as noted above); these bands may have obscured the visualization of a σ or an α subunit. Additional evidence that a σ factor was not immunoprecipitated by the β antibody in bacteria exposed to the cross-linking agent derives from the observation that no new proteins were immunoprecipitated from lysates of chlamydia-infected cells subjected to a heat shock stress (data not shown).

Immunoblots demonstrate that the β subunit protein is detectable early. Figure 6A shows a fluorograph of an SDS-PAGE gel of HeLa cells infected with chlamydiae for various times and pulse-labeled with [35S]methionine in the presence of cycloheximide. Detectable chlamydial protein synthesis was observed at 7 h postinfection (h.p.i.) and continued throughout the life cycle; this finding is consistent with previous studies (25). A doublet of polypeptides of approximately 150 kDa was present at 7 h.p.i.; this doublet had the mobility characteristic of β and β' subunits of procaryotic RNA polymerase. Figure 6B shows an immunoblot of a similar gel; the β polyclonal antiserum recognized a 150-kDa protein in chlamydia-infected HeLa cells as early as 7 h.p.i.; this 150-kDa species was not recognized by preimmune serum (data not shown). A slightly faster migrating band was seen in uninfected host cells with both preimmune (data not shown) and immune sera. An additional faint band was recognized by the immune antiserum specifically in chlamydia-infected cells; this polypeptide may represent a degradation product of the β subunit protein.

DISCUSSION

Characterization of the chlamydial transcriptional apparatus is of interest because prior studies have suggested that chlamydial promoter sequences differ from those previously characterized in other bacteria (9, 24, 29; Engel and Ganem, in press). In this article we describe the cloning and analysis of the β subunit of RNA polymerase from the murine strain of *C. trachomatis*. Using a PCR-based approach, we designed synthetic oligonucleotides to regions of this protein conserved between *E. coli* and plant chloroplast RNA polymerase and used these as primers to amplify the intervening chlamydial sequence. This PCR-generated fragment was then used as a probe to isolate a genomic fragment encoding the β subunit. DNA sequence analysis of this fragment demonstrated that the gene order of the cistron encoding the β and β' subunits of RNA polymerase resembles that of *E.*
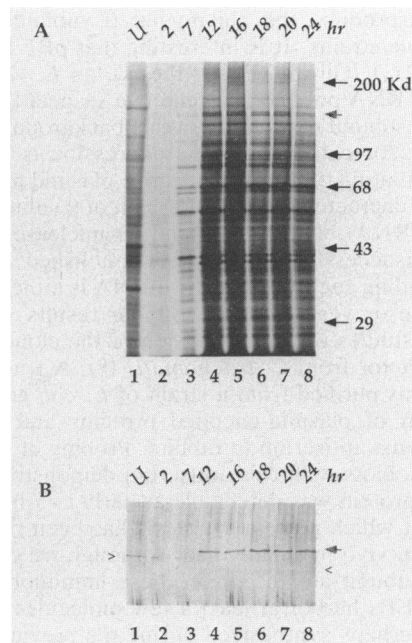

FIG. 6. Developmental immunoblot with the β antibody. Dishes of confluent HeLa cells were infected with chlamydiae for the indicated times and then pulse-labeled with [35S]methionine for 30 min. The cells were lysed in lysis buffer (see Materials and Methods), electrophoresed on a 12% SDS–polyacrylamide gel, and transferred to nitrocellulose filters. (A) Autoradiograph of the filter. Protein sizes (in kilodaltons) are indicated to the right. The shaded arrow indicates the β subunit. (B) Immunoblot of the same filter with the β antiserum. The shaded arrow indicates the β subunit. The > indicates a HeLa cell protein that cross-reacts with the β antisera. The < indicates a possible degradation product of the β subunit. Lane 1, Uninfected HeLa cells. Lanes 2 to 8, HeLa cells infected with chlamydiae for 2, 7, 12, 16, 18, 20, or 24 h, respectively.

coli. A similar strategy was used to isolate the chlamydial α and β' subunit genes by PCR but was unsuccessful. Perhaps the regions from which the degenerate oligonucleotide primers were derived are not conserved in the chlamydial homologs. It is also formally possible that chlamydiae entirely lack an α homolog. Alternatively, some feature of the oligonucleotides (such as secondary structure) may have prevented them from serving as effective primers in the PCR (J. Engel, unpublished observations).

The chlamydial β subunit gene directed the synthesis of a 150-kDa protein product when expressed in a strain of *E. coli* that depended upon thermoinduction for expression of plasmid-encoded genes. Interestingly, pBETA and its derivatives could not be transformed into a strain of *E. coli* (31), where induction, though still dependent on T7 polymerase for expression, is under the control of a *lac* promoter (F. Malik and J. Engel, unpublished observations). The chlamydial β and β' products were not toxic to *E. coli* in the HMS262(pGP1-2) background, as strains containing these constructs could be grown stably at 42°C (F. Malik and J. Engel, unpublished observations). Attempts by others to express in *E. coli* a mutant β subunit of *E. coli* RNA polymerase were successful only when induction was dependent upon heat shock. These workers hypothesized that the high temperature of heat shock denatured the mutant polypeptide, rendering it insoluble and thus unavailable to compete with the wild-type subunit for assembly into the core enzyme (13). Similar explanations may account for our

ability to overproduce the chlamydial β subunit only in thermoinducible strains. It is interesting that pBETA could be cloned in TG1 (Gibson, Ph.D. thesis), an *E. coli* strain lacking the T7 RNA polymerase gene; we suspect that there is very little β subunit expression in this background, where the only route for chlamydial gene expression is by RNA polymerase initiating transcription from a plasmid promoter.

Attempts to characterize the expression of β subunit RNA by Northern (RNA) blot analysis and S1 nuclease analysis have been unsuccessful (J. Engel, unpublished observations). This finding suggests that the mRNA is either of very low abundance or very unstable. Similar results were obtained during studies of the expression of the cloned major vegetative σ factor from *C. trachomatis* (8). A truncated β polypeptide was purified from a strain of *E. coli* geared for overexpression of plasmid-encoded proteins and used to raise a polyclonal antiserum in rabbits. Probing of developmental immunoblots with this antiserum demonstrated that the β subunit protein was detectable as early as 7 h.p.i., the earliest time at which protein synthesis has been reproducibly detected in vivo in chlamydiae. Although we could not detect the β subunit at 2 h.p.i. on these immunoblots, we presume that EBs harbor at least a few molecules of RNA polymerase, perhaps synthesized during the previous cycle of replication. These polymerase molecules, then, would initiate the transcription of the earliest genes during the next round of intracellular chlamydial replication.

The characterization of the transcriptional machinery from other eubacteria has depended on direct biochemical purification of bacterial polymerase combined with assaying the holoenzyme by in vitro transcription on specific templates. This approach is not feasible for chlamydiae, as the organism grows so poorly in culture that it would be exceedingly difficult to generate the necessary starting material for such large-scale enzyme purifications. We initiated this study with the presumption that this β-specific antiserum would be of assistance during the biochemical purification of chlamydial RNA polymerase, for example, for use during an affinity purification procedure. This strategy has been used successfully for the purification of other bacterial RNA polymerases (22, 30). Our results, however, demonstrate that this particular antibody is able to immunoprecipitate only the β subunit of RNA polymerase. Several explanations are possible for this observation. The enzyme may dissociate during the immunoprecipitation under our lysis and wash conditions. Notably, though, washing the immunoprecipitates under gentler conditions (150 mM NaCl) did not allow the immunoprecipitation of intact core enzyme (J. Engel, unpublished observations). Alternatively, this antiserum may only recognize epitopes on unassembled β polypeptide chains or epitopes that are masked when the core or holoenzyme assembles. Relevant to this last point is the fact that the antiserum was raised to the N-terminal portion of the β subunit.

We also asked whether the other subunits of RNA polymerase could be immunoprecipitated if chlamydia-infected cells are exposed to a cross-linking agent prior to immunoprecipitation. While such an approach would not be directly applicable to the purification of functional holoenzyme, it could provide a convenient way to identify alternative σ factors associated with RNA polymerase at various times during the life cycle or under different environmental conditions. We were unable, however, to identify the σ subunit in such immunoprecipitates, even under conditions in which a new σ factor might be expected to stably associate with core enzyme (e.g., heat shock [J. Engel, unpublished observa-

tions] or early in the intracellular developmental life cycle). As the majority of RNA polymerase molecules in a cell exist as core enzyme, we may not be able to detect a σ polypeptide above the background level of other nonspecifically immunoprecipitated proteins. Whether the α subunit was coprecipitated could not be ascertained, because several proteins of ca. 40 kDa were precipitated specifically by the immune serum. One of these proteins is probably the major outer membrane protein, which binds nonspecifically to protein A-Sepharose (Richard Stephens, personal communication) and thus contaminates the precipitates.

Based on our success in expressing the chlamydial β and σ proteins in *E. coli* (8), we are now directing our efforts to cloning and expressing the α and β' subunits in *E. coli* with the ultimate goal of coexpression of all the chlamydial RNA polymerase subunits in *E. coli*. This approach will allow the reconstitution of chlamydial RNA polymerase in *E. coli* for use in in vivo transcription or in vitro transcription assays on authentic chlamydial templates, enabling us to further study the *cis* elements and *trans*-acting factors that underlie the genetic regulation of the chlamydial life cycle.

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