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Characterization of Late Gene Promoters of *Chlamydia trachomatis*

MICHAEL J. FAHR, ANNEMARIE L. DOUGLAS, WEI XIA, AND THOMAS P. HATCH*

Department of Microbiology and Immunology, University of Tennessee, Memphis, Tennessee 38163

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Chlamydiae possess an intracellular developmental cycle defined by the orderly interconversion of infectious, metabolically inactive elementary bodies and noninfectious, dividing reticulate bodies. Only a few stage-specific genes have been cloned and sequenced, including the late-stage cysteine-rich protein operon and two late-stage genes encoding histone-like proteins. The aims of this study were to identify additional late-stage genes of *Chlamydia trachomatis*, analyze the upstream DNA sequence of late genes, and determine the sigma factor requirement of late genes. Stage-specific RNA, made by chlamydiae isolated from host cells, was used to probe *C. trachomatis* genomic libraries. Two new late genes, designated *ltuA* and *ltuB*, were identified, cloned, and sequenced. The predicted peptides encoded by *ltuA* and *ltuB* do not bear strong homology to known proteins, and the function of the new late genes is not known. The 5 ends of the transcripts of *ltuA*, *ltuB*, the cysteine-rich protein operon, and the two histone-like genes (*hctA* and *hctB*) were mapped, and a consensus −10 promoter region of TATAAT was derived from their upstream DNA sequences. In vitro transcription from templates encoding the promoter regions of *ltuA*, *ltuB*, and *hctA* cloned into the transcription assay vector pUC19-*spf* was found to be strongly stimulated by the addition of recombinant chlamydial σ^{66}, while transcription from the putative *hctB* promoter region cloned in pUC19-*spf* was not detected in either the presence or absence of added σ^{66}. These results suggest that the transcription of at least some chlamydial late-stage genes is dependent on σ^{66}, which is homologous to the major sigma factors of other eubacteria.

Members of the genus *Chlamydia* are obligate intracellular parasites which progress through a biphasic developmental cycle (recently reviewed in references 5, 29, and 31). The infectious form, called an elementary body (EB), possesses an electron-dense nucleoid and is metabolically inert. The cycle is initiated by the attachment of EBs to host eucaryotic cells and the subsequent endocytic uptake of the EBs. By 4 to 8 h postinfection (p.i.), conversion of the EB form to the noninfectious, dividing reticulate body (RB) form is complete, marking the end of the early stage. RBs possess DNA that is relatively evenly distributed throughout the cytoplasm, and they synthesize DNA, RNA, and protein. Throughout the middle of the developmental cycle, RBs divide by binary fission within a membrane-bound vacuole called an inclusion, which increases in size proportionally to the number of chlamydiae within. As early as 18 to 20 h p.i., some RBs begin to reorganize back to EBs, marking the beginning of the late stage. A few infectious EBs can be isolated from infected cells between 20 and 24 h p.i.; however, the cycle becomes increasingly asynchronous with time, with morphological forms resembling RBs, intermediate reorganizing forms, and EBs being present within the same vacuole. In most tissue culture systems, cells infected with moderate numbers of chlamydiae (five or fewer) will continue to support parasite growth and reorganization until 30 to 72 h p.i. Eventually, the infected host cell cytoplasm becomes almost completely filled with one or more chlamydia-laden vacuoles and the host cell and inclusion membranes lyse, releasing a mixture of infectious EBs and noninfectious forms.

The factors that limit chlamydiae to an intracellular lifestyle have not been completely delineated. One important factor appears to be their requirement for host-supplied nucleoside triphosphates (NTPs) (29). Similarly, signals that trigger metabolic activity in intracellular EBs early in the cycle and the reorganization of RBs late in the cycle have not been identified. Within 1 to 2 h p.i., synthesis of many chlamydial proteins can be detected by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (35). Many of these proteins are likely to have housekeeping functions. A few, however, appear to be preferentially made during the early phase of the cycle (35). Only one early gene has been cloned and sequenced to date, and its function is not known (42). Four late genes that are not expressed until RBs begin to reorganize to EBs have been cloned and sequenced. Two of these, *omp3* and *omp2*, make up an operon which encodes the small and large cysteine-rich proteins (CRPs) (1, 2, 25). The other two late genes (*hctA* and *hctB*) encode histone-like proteins which are believed to bind to chlamydial DNA and to be responsible for the nucleoid structure seen in EBs (4, 7, 19, 33, 34, 41).

Two general mechanisms of regulation of transcription initiation of any given stage-specific gene can be envisioned: (i) recognition of a stage-specific promoter by a stage-specific sigma factor (with or without auxiliary transcription factors) in a manner analogous to that in sporulation in *Bacillus subtilis* (20), and (ii) recognition of the promoter by the major sigma factor, with stage-specific regulation at the level of repression and activation by auxiliary transcription factors. To date, only one chlamydial sigma factor, designated SigA or σ^{66}, has been identified (12, 14, 24), although others may exist. On the basis of its homology to the conserved regions of the σ^{70} family of bacterial sigma factors (26) and its expression throughout the chlamydial developmental cycle (24), σ^{66} is considered to be the major sigma factor of chlamydiae.

Transient transformation of chlamydiae has been reported (40); however, a method for stable transformation and a usable genetic system have not been developed. Our laboratory, therefore, has developed an in vitro transcription system to investigate mechanisms of transcriptional regulation in chlamydiae (12, 28). The system includes a partially purified preparation of chlamydial RNA polymerase, recombinant σ^{66}, a cloned chlamydial promoter as template, and NTP substrates

* Corresponding author. Mailing address: Department of Microbiology, University of Tennessee, 858 Madison Ave., Memphis, TN 38163. Phone: (901) 448-4664. Fax: (901) 448-8462. Electronic mail address: THATCH@UTMEM1.UTMEM.edu.

(including [α-^{32}P]UTP). Enhancement of transcription by supplemental σ^{66} indicates that the cloned promoter is recognized by this sigma factor. We have used this system to demonstrate that the antisense transcripts of the cryptic chlamydial plasmid and one of the major outer membrane protein (MOMP) gene promoters requires σ^{66} for optimal activity (11, 12). We have also used a monoclonal antibody that reacts with σ^{66} on Western immunoblots to inhibit in vitro transcription of the CRP operon, suggesting that at least one late-stage promoter is recognized by σ^{66} (28). The sigma factor requirement of the other known late genes is not known.

The purpose of the current investigation was threefold: (i) to clone and sequence additional late-stage *C. trachomatis* genes; (ii) to map the 5′ ends of known late-gene transcripts in order to identify putative promoter regions; and (iii) to further define the role of the major sigma factor in late-stage gene transcription. We report the identification of two new late genes and a −10 promoter consensus for late-stage genes of TATAAT, and we demonstrate that the new late genes and *hctA*, in addition to the previously reported CRP operon (28), require σ^{66} for expression in vitro.

MATERIALS AND METHODS

Host-free assays. *C. trachomatis* LGV434, serovar L2, was propagated in HeLa 229 cells, and host-free RBs were harvested from 2×10^8 infected cells and purified by Renografin centrifugation (10). Host-free RNA probes were generated by incubating 14-, 16-, or 30-h RBs for 30 min at 37°C in 0.6 ml of Dulbecco phosphate-buffered saline (GIBCO, Grand Island, N.Y.) containing 1 mM dithiotheitol, 100 μM each ATP, UTP, and CTP, and 250 μCi of [α-^{32}P]GTP (800 Ci/mmol; NEN Research Products, Boston, Mass.), and RNA was isolated by the hot-acid-phenol method as described by Crenshaw et al. (10).

Isolation of late-stage clones. A partial genomic library of *C. trachomatis* L2 was constructed by the ligation of 1- to 5-kb fragments from a partial *Sau*3A digest into the *Bam*HI site of pBluescript KS(+) (Stratagene, La Jolla, Calif.). A complete genomic library was constructed by partial digestion of genomic DNA with *Hae*III and *Rsa*I and ligation of 1- to 5-kb fragments into pUC19. Recombinant plasmids were transformed into *Escherichia coli* MC1061. Duplicate colony blots of the libraries were prepared on MSI Nitroplus nylon filters (Micron Separations, Westboro, Mass.), as described by Maniatis et al. (27), and probed with 14- and 30-h radiolabelled host-free RNA. The filters were prehybridized in a solution consisting of 50% formamide, 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 5× Denhardt's solution (2% bovine serum albumin, 2% Ficoll-400, 2% polyvinylpyrrolidone), 100 μg of calf thymus DNA per ml, and 1% glycine. Hybridizations were carried out overnight at 42°C in 50% formamide–5× SSC–1× Denhardt's solution–100 μg of calf thymus DNA per ml–0.3% SDS. Following hybridization, the blots were washed three times in 2× SSC–0.2% SDS for 20 min at 42°C. Clones which reacted more strongly with 30-h RNA than with 14-h RNA were identified and subjected to a second screening by a dot-blot procedure. DNA was isolated from the clones and denatured with 0.3 M NaOH for 30 min at room temperature. The samples were chilled to 4°C, diluted with an equal volume of 2 M ammonium acetate, loaded in duplicate onto MSI Nitroplus nylon filters using a Minifold I apparatus (Schleicher & Schuell, Keene, N.H.), and probed with 16- and 30-h radiolabelled host-free RNA.

Other nucleic acid manipulations. The oligonucleotides used in this study are shown in Table 1. DNA sequencing was performed with a Sequenase 2.0 kit (U.S. Biochemical Corp., Cleveland, Ohio), and primer extension analysis of RNA isolated at 26 h p.i. was performed as described by Kingston (23).

Northern (RNA) blots were carried out by the formaldehyde method described by Maniatis et al. (27). Plasmid DNA probes were radiolabelled with [α-^{32}P]dCTP (800 Ci/mmol; NEN) by the method of Feinberg and Vogelstein (18), using a random-primed DNA-labeling kit (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). Oligonucleotides were 5′ end labelled with 125 μCi of [γ-^{32}P]ATP (600 Ci/mmol; NEN) as described previously (17). Hybridization with plasmid DNA probes was carried out as described for host-free RNA probes. When blots were probed with labelled oligonucleotides, the preincubation conditions were 42°C for 3 h in a solution containing 6× SSC, 0.05% sodium PP_i, 5× Denhardt's solution, 100 μg of calf thymus DNA per ml, and 0.5% SDS; hybridization was carried out overnight at 42°C in the same solution except that 1× Denhardt's solution was used, and washing was carried out as described for host-free RNA probes.

cDNA synthesis for reverse transcriptase-PCR analysis of RNA was carried out as described by Kingston (23), with chlamydial RNA (20 μg per sample) isolated at 30 h p.i. from purified RBs, avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim), and 100 ng of oligonucleotide primer O-699 (Table 1). Control samples were treated with 1 μg of RNase A prior to cDNA synthesis. After DNA synthesis, the reaction mixtures were incubated with 1 μg of RNase A at 37°C for 30 min and heated at 60°C for 5 min, and DNA was extracted with phenol-chloroform (1:1, vol/vol) and precipitated with ethanol. PCR was performed with a DNA thermal cycler (Perkin-Elmer Cetus, Norwalk, Conn.) under the following conditions: 25 cycles at 94°C for 1 min to denature the DNA, 50°C for 2 min to anneal primers to the template, and 72°C for 1 min for the polymerization step. The reaction mixture consisted of cDNA product as template, 100 ng each of oligonucleotides O-358 and O-699, 1 U of *Taq* polymerase (Perkin-Elmer Cetus), 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% (wt/vol) gelatin (Sigma), and 250 μM each dATP, dCTP, dGTP, and TTP (Pharmacia LKB Biotechnology, Piscataway, N.J.) in a final volume of 100 μl.

TABLE 1. Oligonucleotides used in this study

Gene	Oligonucleotide	Sequence[a]
hctA	O-584	5′-GCCGTATCTTTTAGCGCCAT
	L18KH-1	5′-GCGATCTAGACTCTTCAAAGAAGACTGCAAAG
	L18KH-2	5′-GCGAGGATCCCCTCTAGTTGTTGTTTTAAAC
hctB	O-925	5′-TGTACTCCCAACATGTTCAT
	L26K-1	5′-GCGATCTAGACCTCGAAGACAATCCAGTAGC
	L26K-2	5′-GCGAGGATCCCTACTACTTATTTGATCTATCG
	L26K-3	5′-GCGAGGATCCGATCTATCGACAAGGAGAATG
ltuA	O-51	5′-CTACAGGTGCCAATAAACCG
	L47-1	5′-GCGATCTAGAGGGAACAGTCCTTCTTCGAT
	L47-2	5′-GCGAGGATCCAAGAGAGAAGTGATAGACAG
ltuB	O-358	5′-CATCATTACAGCACAGCCTC
	O-503	5′-CAATAAAGTGCATCTCTGTA
	O-581	5′-GAGGCTGTGCTGTAATGATG
	O-699	5′-ATATATACACCCAGGCTCCC
	LT1-1	5′-GCGATCTAGAGAGCACTGATGAGAGGGAACG
	LT1-2	5′-GCGAGGATCCCATGAGCAGAAACCAGAATAA
spf	spf	5′-GGCGGCTAAAATATTCAGCCA

[a] Underlining designates 5′ extensions of gene sequences consisting of endonuclease restriction sites and other bases.

RNA-capping reactions were conducted as described by Stoddard and Howe (39), with 200 μg of RNA from infected cells isolated by the hot-acid-phenol method, 0.5 mCi of [α-^{32}P]GTP (3,000 Ci/mmol; NEN), and 8 U of guanylyltransferase (Bethesda Research Laboratories, Gaithersburg, Md.). Southern blots (37) of pMJF15, pMJF35, and pMJF31 DNA treated with *Eco*RI, *Pst*I-*Kpn*I, and *Hin*cII-*Xba*I, respectively, were probed with capped RNA under the hybridization conditions described for host-free RNA probes. After exposure to autoradiographic film, the blots were washed twice in RNase digestion buffer (10 mM Tris-HCl [pH 7.5], 300 mM NaCl, 5 mM EDTA), treated with 40 μg of RNase A (Sigma Chemical Co., St. Louis, Mo.) per ml at 37°C for 30 min, and reexposed to autoradiographic film.

Description of clones. pFEN50 (3), encoding the complete *C. trachomatis* L2 MOMP gene (*omp1*), was kindly supplied by Francis E. Nano, University of Victoria, Victoria, British Columbia, Canada. pCT47 (10) encodes part of a *C. trachomatis* L2 rRNA operon. Clones encoding the inserts of pMJF15 and pMJF60 were isolated by screening the *Hae*III-*Rsa*I library with host-free RNA. Inserts were recloned into the *Bam*HI-*Eco*RI sites of pBluescript KS(+). pMJF15 contains a 2.1-kb insert encoding the first 74 amino acids of the small CRP and approximately 1.8 kb of upstream sequence. pMJF60 contains a 1.5-kb insert encoding a late-stage transcript of about 250 bases and an incomplete upstream open reading frame (ORF) with homology to phosphoglucoisomerase. The late transcript encodes a short ORF of 47 codons.

pMJF30 was isolated from the *Sau*3A partial genomic library by using host-free RNA probes. It contains a 2.1-kb insert encoding a late transcript designated T2. pMJF31 is a subclone of pMJF30 containing a 740-bp *Hin*cII-*Xba*I insert cloned into pBluescript KS(+); it encodes the 5′ end of T2. pMJF32 contains a 2.3-kb *Pst*I fragment of a *C. trachomatis* L2 genomic digest cloned into pBluescript KS(+). The fragment was identified on the basis of hybridization to O-699; it encodes both transcripts T1 and T2. pMJF38 consists of a 1.1-kb *Bgl*II fragment of pMJF32 cloned into the *Bam*HI site of pBluescript KS(+). pMJF35 consists of an 874-bp *Pst*I (from the multiple-cloning site)-*Xmn*I fragment of pMJF38 subcloned into pBluescript KS(+); it encodes the 5′ end of T1 and lacks T2-encoding sequence.

The promoter regions of late-stage genes were cloned into the *Bam*HI-*Xba*I sites of transcription assay vector pUC19-*spf*′ (16) with the exception of

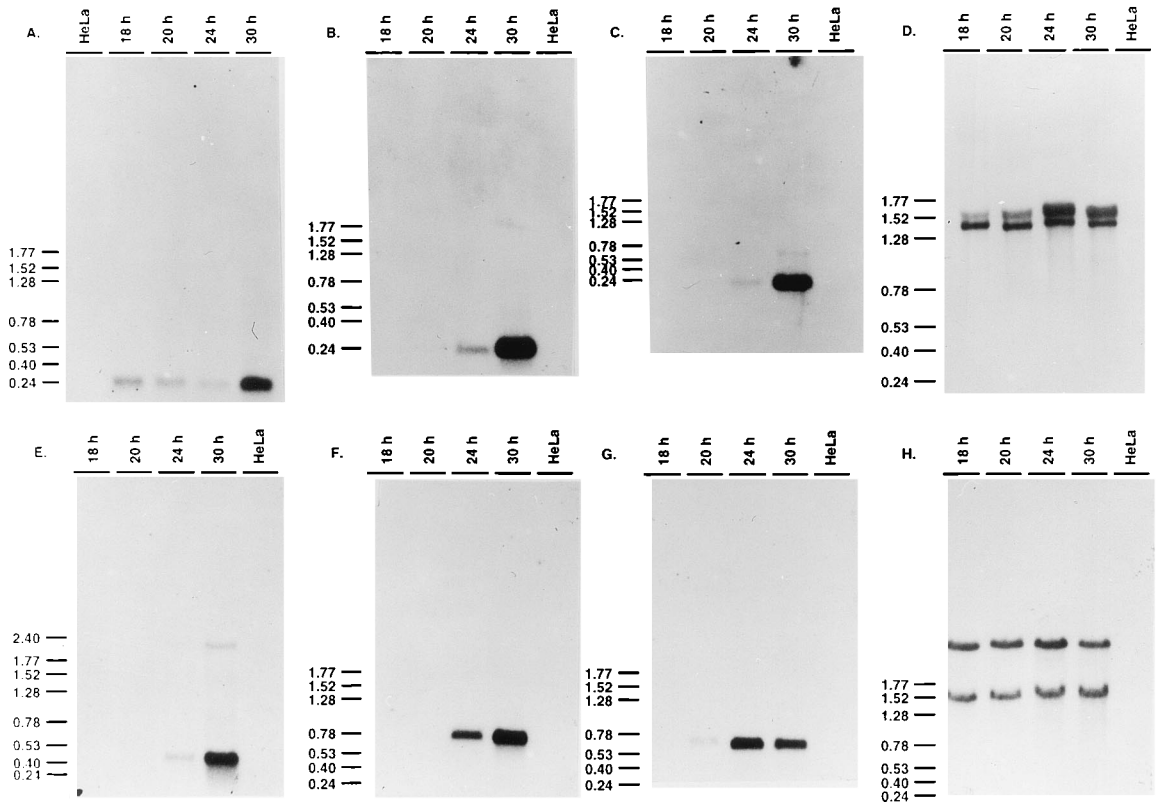

FIG. 1. Northern blots of late clones. RNA was isolated from *C. trachomatis*-infected cells at 18, 20, 24, and 30 h p.i. and from uninfected HeLa cells. The same preparation of chlamydial RNA was used for all blots, and the amount of RNA analyzed was adjusted so that the signals to the rRNA probe (H) were equal at all times. Also shown is a blot probed with *omp1* (MOMP gene [D]). *omp1* is not a late gene; it encodes two transcripts, the shorter of which predominates during the earlier stages of the developmental cycle (38). Probes used were as follows: (A) O-51, an oligonucleotide based on pMJF60 sequence; (B) pMJF30; (C) pMJF32; (D), pFEN50 (encodes *omp1*); (E) pMJF15 (CRP operon); (F) O-925 (an oligonucleotide based on *hctB* sequence); (G) O-584 (an oligonucleotide based on *hctA* sequence); (H) pCT47 (rRNA operon). The migration of RNA standards (in kilobases) is shown to the left of each blot.

pALR212, which was blunt-end ligated into the *Hin*cII site. The promoter inserts were generated by PCR amplification of *C. trachomatis* L2 genomic DNA and forward and reverse primers (Table 1) as follows: pALR211 (*ltuB*), oligonucleotides LT1-1 and LT1-2; pALR212 (*hctA*), oligonucleotides L18KH-1 and L18KH-2; pALR213 (*ltuA*), oligonucleotides L47-1 and L47-2; pALR214 (*hctB*), oligonucleotides L26K-1 and L26K-2; and pALR214B (*hctB*), oligonucleotides L26K-1 and L26K-3. The DNA sequence of all inserts was determined and found to be identical to that of the primary late-gene clones.

In vitro transcription assays. Transcripts were generated in vitro from the pALR templates described above. Chlamydial RNA polymerase preparations were made from 26-h p.i. RBs, and recombinant σ^{66} was prepared as described by Douglas et al. (12). All procedures for the generation and assay of transcripts labelled with [α-^{32}P]UTP are also described by Douglas et al. (12). Primer extension analysis of in vitro transcripts was carried out as described by Mathews et al. (28), except that an oligonucleotide complementary to the *spf* gene (Table 1) was used as primer.

Nucleotide sequence accession numbers. The nucleotide sequences of *ltuA* and *ltuB* are available from GenBank (L40822 and L40838, respectively).

RESULTS

Use of host-free RNA to screen for late-stage-specific genes. Following the method of Crenshaw et al. (10), we used radiolabelled RNA made by *C. trachomatis* L2 RBs isolated at 14 and 30 h p.i. to probe duplicate colony blots of two *C. trachomatis* L2 genomic libraries in an attempt to identify late-stage-specific clones. Several late clones were identified and confirmed to encode late genes, including two previously identified late genes, the CRP operon and *hctB*, and two new late transcription units. The results of a Northern blot analysis of RNA from cells infected with *C. trachomatis* for 18, 20, 24, and 30 h and probed with labelled insert (or oligonucleotides based on insert DNA) of the new clones (pMJF60 and pMJF30), known late chlamydial genes (the CRP operon, *hctA*, and *hctB*), a non-late gene (*omp1*), and a gene encoding chlamydial rRNA are shown in Fig. 1. The blots shown in Fig. 1A and B indicate that pMJF60 and pMJF30 encode all or parts of short transcripts of 250 nucleotides or less. The pMJF60 transcript was detected as early as 18 h p.i. but was found in much larger amounts at 30 h than at earlier times; consequently, we have designated the gene encoding this late transcription unit *ltuA*. The small 240-nucleotide transcript encoded by pMJF30, which we have designated transcript T2, was barely detectable at 24 h p.i. and accumulated in large amounts at 30 h p.i. following a pattern similar to that seen for the CRP operon and *hctB*. pMJF30 also encodes part of a second late transcript of 550 bases, designated transcript T1, which was not detected on the blot shown in Fig. 1B but was detected by using the overlapping clone pMJF32 (Fig. 1C). We have found that the small T2 transcript is a posttranscriptional processing product of the larger T1 transcript (see below) and have designated the gene encoding the primary T1 transcript *ltuB*.

It is interesting that the genes encoding the two histone-like proteins (*hctA* and *hctB*) were not found to share a common pattern of expression: *hctA* RNA was first detected at 20 h and peaked at 24 h p.i. (Fig. 1G), while *hctB* RNA was first observed at 24 h and was present in greatest amount at 30 h p.i. (Fig. 1F). This observation is consistent with the conclusion that these genes are not part of a common operon, as was previously determined by a chromosome-mapping study (6).

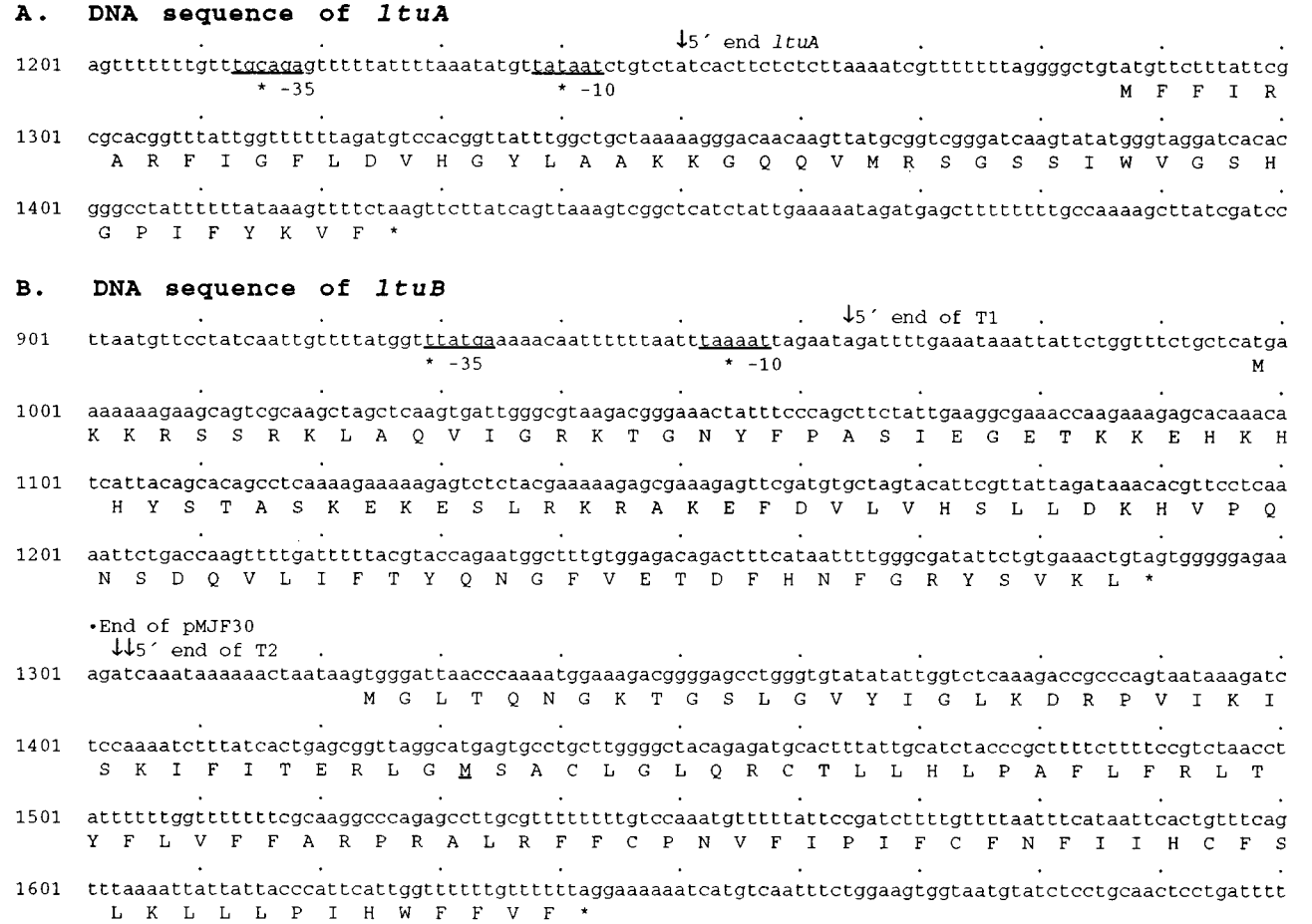

FIG. 2. DNA sequence of *ltuA* (A) and *ltuB* (B). 5′ ends of transcripts, as identified by primer extension (↓), and the end of pMJF30 ( ) are indicated above the sequence. Proposed −10 and −35 promoter sequences are underlined. Numbering of the sequences is the same as that submitted to GenBank/EMBL data bases.

The sizes of the histone gene transcripts, about 700 to 750 bases for *hctB* and 600 for *hctA*, are consistent with previous reports (19, 33) and suggest that these transcripts are monocistronic. Two transcripts were noted when the blots were probed with both CRP operon DNA and *omp1* DNA, as previously reported (25, 38) (Fig. 1D and E).

DNA sequence of *ltuA* and *ltuB*. When the DNA sequence of the insert of pMJF60 was determined, a 47-codon ORF (ORF47) was identified (Fig. 2A). Since the oligonucleotide (O-51) which identified the late transcription unit shown in Fig. 1A is complementary to the mRNA for ORF47, *ltuA* encodes this ORF. The potential peptide encoded by ORF47 does not bear strong similarity to peptides deposited in the Swiss Protein and GenBank/EMBL databases. The 3′ end of a second, partial ORF of 330 codons was located 220 bp upstream of the 47-codon translation start site (sequence not shown; see GenBank accession number L40822). The predicted amino acid sequence of the partial ORF is between 31 and 35% identical to phosphoglucoisomerase of several organisms. A housekeeping gene like the isomerase would be expected to be most actively transcribed during the early and middle phases of the developmental cycle. However, as has been the case with several other chlamydial genes (15, 17, 42), attempts to detect the isomerase-like gene transcripts on Northern blots of midcycle and late-stage RNA (in our case, using an oligonucleotide probe) were unsuccessful (data not shown).

The 5′ end of late clone pMJF30 is shown in Fig. 2B. The longest ORF (107 codons) predicted within pMJF30 is initiated with a GTG codon and contains 16 phenylalanine residues; a smaller ORF (72 codons) that is initiated with ATG is contained within this ORF. Primer extension analysis, using the complementary oligonucleotide O-503, indicated that the 5′ end of the 240-base T2 transcript made by pMJF30 is encoded from sequence within 2 to 3 bp of the end of the clone (Fig. 2B and data not shown). Because a primary objective of this study was to characterize late-stage promoters, it was important to obtain sequence upstream of the T2 transcript. We therefore used an oligonucleotide based on sequence close to the 5′ end of T2 (O-699) to probe a Southern blot of *C. trachomatis* genomic DNA digested with various restriction endonucleases. A reactive 2.1-kb *Pst*I fragment was used to construct pMJF32, which encodes transcript T2 and the larger 550-base transcript designated T1 (Fig. 1C). pMJF32 also encodes a second short ORF of 98 codons, located just upstream of the ORF encoded by transcript T2 (Fig. 2B). Neither the 107-codon nor the 98-codon ORF bears strong homology to proteins in the Swiss Protein or GenBank/EMBL databases, although a potential ATP/GTP-binding site is present near the N terminus of the longer predicted peptide.

Posttranscriptional processing of the primary transcript of *ltuB*. Because we were interested in identifying late gene promoters, it was important to determine the relationship between transcripts T1 and T2. In particular, it was important to deter-

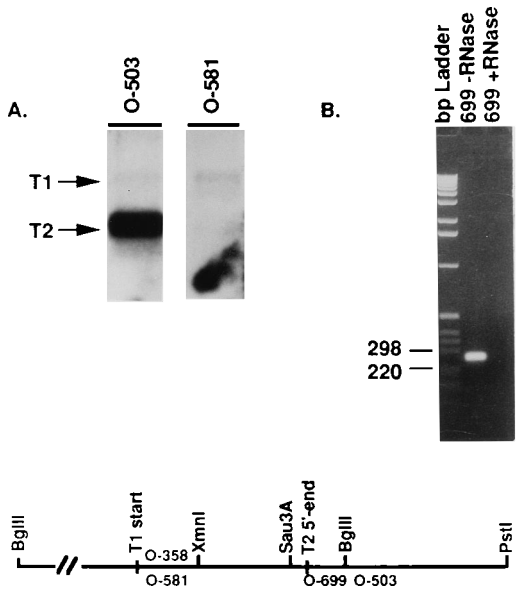

FIG. 3. Analysis of T1 and T2 transcripts of *ltuB*. (A) A Northern blot of 30-h *C. trachomatis* RNA was probed with oligonucleotides O-503 and O-581. O-503 is complementary to the 5′ end of T2, and oligonucleotide O-581 is based on upstream sequence of the same strand of DNA. Both T1 and T2 were identified with O-503, indicating that T1 and T2 are encoded by the same strand of DNA. (B) Reverse transcriptase-PCR analysis of 30-h RNA. cDNA was first generated with reverse transcriptase and primer O-699. A PCR fragment was then generated with the forward primer O-358 and the reverse primer O-699. A fragment of the size predicted (270 bp) if the T1 and T2 transcripts overlap was generated (lane -RNase). The fragment was not the result of amplification of any genomic DNA in the RNA preparation, because it was not generated when the RNA sample was treated with RNase prior to synthesis of cDNA (lane +RNase). The positions of the primers are shown in the map located at the bottom of the figure.

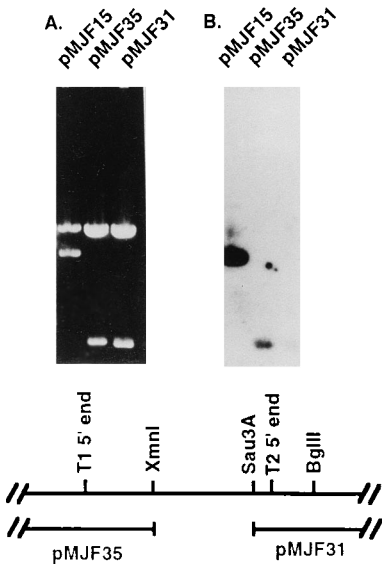

FIG. 4. Capping of the 5′ end of *ltuB* primary transcripts. Clones pMJF15 (encoding the 5′ end of the CRP operon), pMJF35 (encoding the 5′ end of T1), and pMJF31 (encoding the 5′ end of T2) were treated with restriction endonucleases that excised inserts (lower bands) from vectors (upper bands). The fragments were separated on an agarose gel and analyzed by Southern blot, with chlamydial RNA capped with [α-^{32}P]GTP as the probe. (A) Ethidium bromide stain of the gel; (B) autoradiograph of the blot. Strong signals were noted only to fragments of clones encoding the 5′ ends of primary transcripts (pMJF15 and pMJF35). The positions of clones pMJF35 and pMJF31, relative to the 5′ ends of T1 and T2, are shown at the bottom of the figure.

mine whether both T1 and T2 were primary transcripts or whether T2 was a processed product of the longer T1 transcript. We first determined that T1 and T2 are encoded by the same strand of DNA by probing Northern blots with oligonucleotides: O-581, based on T1 sequence, reacted only with the longer T1 transcript, while O-503, based on T2 sequence, reacted with both T1 and T2 (Fig. 3A). The ratio of T1 to T2, as determined from the blot probed with O-503, appeared to be at least 1:100. We used reverse transcriptase-PCR technology to determine whether T1 overlaps T2. cDNA was first generated from the 5′ end of T2 RNA with reverse transcriptase and with the complementary oligonucleotide O-699 as primer. We then demonstrated that T1 overlaps T2 by generating a predicted 270-bp PCR fragment from the 5′ region of T1 to the 5′ region of T2, using a reverse primer (O-358) based on T1 sequence and O-699 (Fig. 3B).

To determine whether T1 and T2 are primary transcripts, we attempted to label their 5′ ends with [α-^{32}P]GTP, using the eucaryotic capping enzyme guanylyltransferase. Guanylyltransferase specifically recognizes the tri- and diphosphate 5′ ends of primary transcripts and will not label processed transcripts which lack these ends. Late-stage RNA was capped and then used to probe a Southern blot of DNA encoding the 5′ end of T1 (pMJF35), the 5′ end of T2 (pMJF31), and, as a control, the 5′ end of the CRP operon (pMJF15) (Fig. 4). Strong hybridization signals to the inserts of pMJF35 and pMJF15 were noted (Fig. 4B), and these signals were protected when the blots were treated with RNase (data not shown), indicating that pMJF35 and pMJF15 encode the 5′ ends of primary transcripts. A weak hybridization signal to the insert of pMJF31 was also noted (Fig. 4B). However, this was not protected when the blot was treated with RNase (data not shown), indicating that pMJF31 does not encode the start site of a primary transcript. It follows, therefore, that T2 is a stable degradation product of the overlapping primary transcript T1. There appears to be an efficient processing site between the 5′ ends of T1 and T2, since the ratio of full-length T1 to T2 is about 1:100, as noted on the Northern blots shown in Fig. 1C and 3A. At least some portion of the 5′ fragment released from T1 must be relatively stable, since it seems to be considerably more abundant than full-length T1, as indicated by the intense hybridization of capped RNA to pMJF35 compared with pMJF31 (Fig. 4B).

Identification of the 5 ends of late genes and promoter analysis. The 5′ ends of *ltuA*, *ltuB*, and the three previously described late genes were determined by primer extension (Fig. 5). The 5′ end of the CRP operon that we identified (Fig. 5E) was the same as the major end identified by Lambden et al. (25). We noted a strong primer extension signal for *hctB* at the same location that was reported by Brickman et al. (7) (data not shown), as well as a fainter signal 35 bp upstream (Fig. 5D). Both potential 5′ ends are close to inverted repeats; thus, the possibility that the primer extension signals were caused by premature termination of the extension reaction at stem-loop structures in the RNA cannot be ruled out. It is also possible that the *hctB* transcript has two promoters or is processed in a manner similar to the T1 transcript. In the absence of any evidence for processing and because we detected only the 5′ distal signal when we mapped the ends of *hctB* transcripts made in vitro (see below), we chose the 5′ end identified in the experiment shown in Fig. 5D as the putative start site of *hctB*. The 5′ ends of the *C. trachomatis hctA* gene that we report (Fig. 5C) appear to be in the region reported by Kaul et al. (22) for the 5′ end of the *hctA* homolog of *C. psittaci.*

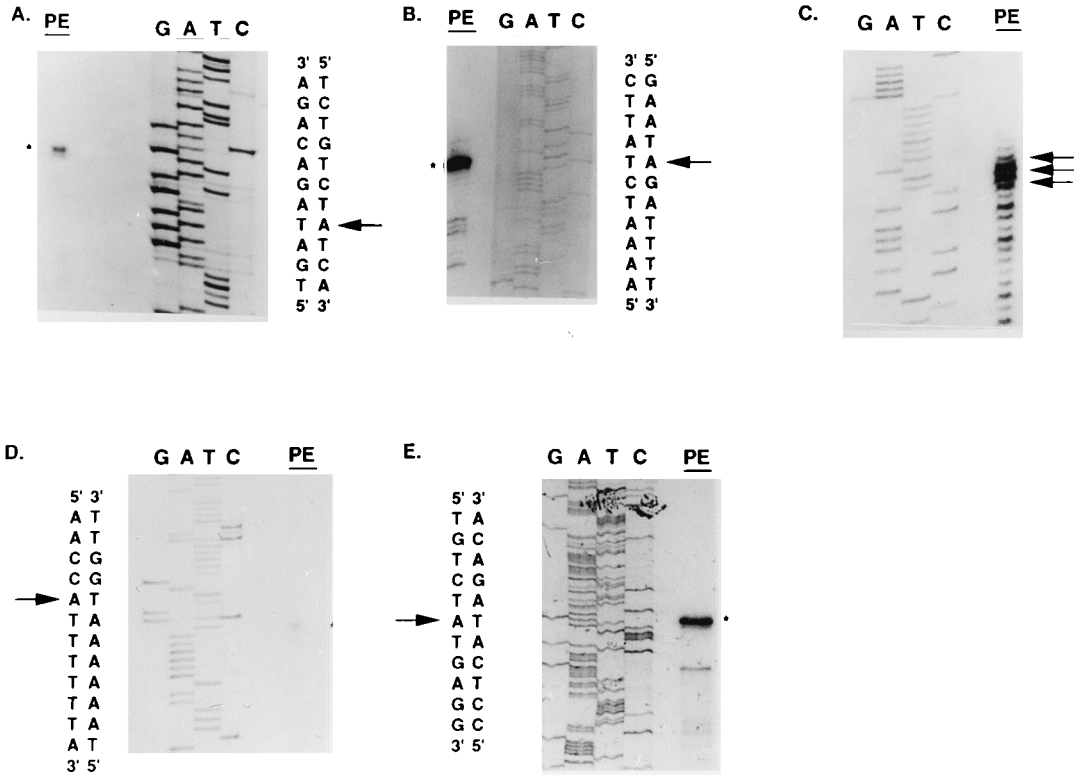

FIG. 5. Primer extension analysis of *C. trachomatis* genes. (A) *ltuA*; (B) *ltuB*; (C) *hctA*; (D) *hctB*; (E) CRP operon. The primer extension products (PE) are noted with an asterisk. DNA sequencing ladders, using the same primer as was used for primer extension, were used to determine the 5′ ends of all genes except *hctA*. For *hctA*, the lengths of the extension product from O-584 were estimated with the sequencing ladder of another gene. The 5′ ends so identified (TTA) are 50, 49, and 48 bp upstream from the AUG translation initiation codon.

The potential transcription start sites, the −10 hexamers of the putative promoters, and sequences extending from bp −90 to +25 of the late genes are shown in Fig. 6. All of the late gene −10 hexamers are centered at or within 1 bp of −10 (relative to the transcription start site) and match the *E. coli* major sigma consensus recognition sequence in a minimum of four positions, with the *ltuA* hexamer being identical in all six positions. Furthermore, the consensus of the −10 region of the late genes is TATAAT. It seems likely, then, that at least some late-stage chlamydial genes are recognized by the same sigma factor, most probably σ^{66}. The putative promoter regions of all of the *C. trachomatis* nonlate genes for which 5′-end data are available, with the exception of *dnaK* and the P1 promoter of *omp1*, are also shown in Fig. 6. *dnaK* was not included, because it is a stress response gene and may be subject to unusual mechanisms of regulation (15), and the P1 promoter of *omp1* is not recognized by σ^{66} in vitro (11). Excluding *omp1* P1 and *dnaK* from the analysis, the consensus −10 hexamer for the nonlate genes is TATANT—although individual promoters often diverge significantly from the *E. coli* consensus.

An attempt was made to identify an *E. coli*-like −35 hexamer (TTGACA) within the upstream regions of the genes shown in Fig. 6. A match in four or more positions with a spacing upstream from the −10 hexamer of between 16 and 19 bp was noted in only five genes: the CRP operon; *omp1* P2 (38); the 70-kDa S1 ribosomal protein gene (36); ORF3, a potential membrane protein encoded by the *C. trachomatis* plasmid (9); and the plasmid antisense transcripts, PT1-PT2 (17).

The late gene sequences from −90 to +25 were scanned for common elements that might represent a late gene signature, and a TNGTT box was noted at least once upstream and once downstream from the start site of all five genes. Multiple copies of the TNGTT box were noted in upstream regions of only one nonlate gene, the *spc* ribosomal protein operon (21), and a single copy of the box was noted downstream of the start site of the S1 ribosomal protein gene; the remaining nonlate genes lacked TNGTT boxes.

In vitro transcription analysis of late genes. The resemblance of the putative −10 regions of the late gene promoters to the *E. coli* major sigma factor consensus hexamer and the high degree of homology between σ^{66} and the major sigma factors of other bacteria (26) suggest that σ^{66} may be responsible for initiating transcription of these genes. We recently developed an in vitro transcription system which was used to demonstrate that transcription of the CRP operon is, in fact, dependent on σ^{66} (28). We used this system to prepare in vitro transcripts of the other chlamydial late genes, *ltuA*, *ltuB*, *hctA*, and *hctB*. DNA templates were constructed for this purpose by cloning PCR fragments of promoter regions identified by primer extension analysis (Fig. 5) into the transcription assay vector, pUC19-*spf*′. Transcripts, initiated from the chlamydial promoters and terminated at the *E. coli spf* terminator in the vector, were generated with [α-^{32}P]UTP, unlabelled NTPs, and partially purified chlamydial RNA polymerase, with and without the addition of recombinant σ^{66}. When recombinant σ^{66} was present in the reaction mixture, templates encoding the promoter regions of *ltuA*, *ltuB*, and *hctA* generated six to seven tandem in vitro transcripts, indicative of imprecise termination at the *spf* terminator (12) (Fig. 7). These signals all were

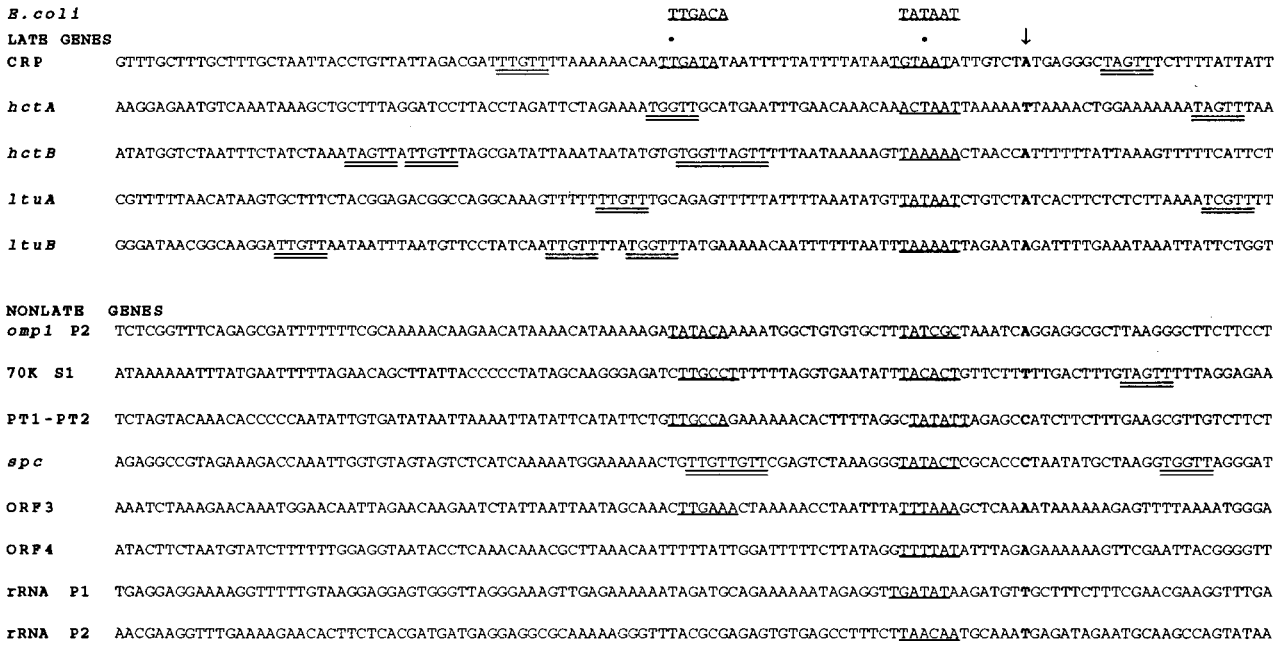

FIG. 6. Putative promoter regions of *C. trachomatis* genes. Late gene 5′ ends (shown in bold and indicated by the arrow) are as identified in Fig. 5. The 5′ ends of nonlate genes are as described by others (*omp1* [38]; 70 kDa S1 ribosomal protein gene [36]; the plasmid antisense transcripts PT1-PT2 [17]; the *spc* ribosomal protein operon [21]; plasmid ORFs 3 and 4 [9]; and the rRNA operon [13]). The sequence shown for the rRNA operon was determined for the mouse pneumonitis strain, while the others were determined for the L2 serovar of *C. trachomatis*. Putative −10 and −35 hexamers are indicated by a single underline, and TNGTT boxes are indicated by double underlines.

greatly reduced when sigma factor was omitted from the reaction, suggesting that the *ltuA*, *ltuB*, and *hctA* promoters are dependent on σ^{66} for initiation of transcription.

Primer extension analysis of *ltuA*, *ltuB*, and *hctA* transcripts generated in the presence of σ^{66} identified the same 5′ ends as were identified for in vivo transcripts (data not shown), supporting the conclusion that σ^{66} recognizes the same promoters in vitro as it does in vivo. We cloned two different possible promoter segments of the *hctB* gene into the pUC19-*spf*′ transcription assay vector: one with sequence from bp −214 to +39 and one from bp −214 to +52, relative to the start site shown in Fig. 6. The former clone excludes the start site reported by Brickman et al. (7), while the latter includes this site. Neither one of these templates generated promoter-specific, radiolabelled transcripts that could be detected on DNA sequencing gels, even in the presence of σ^{66}. However, we were able to detect transcripts generated in vitro from both *hctB* templates with and without added recombinant sigma factor by using the primer extension assay, and the 5′ end that was identified was the same as that identified for RNA made in infected HeLa cells (data not shown). Unfortunately, our primer extension assay is not quantitative; therefore, no conclusions can be drawn regarding the requirement of σ^{66} for transcription of *hctB*.

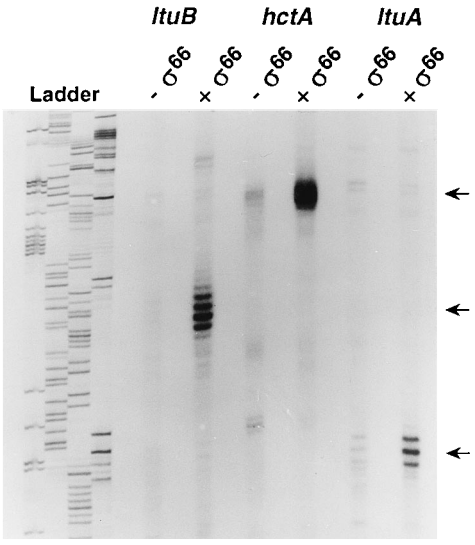

FIG. 7. In vitro transcription of late genes. Radiolabelled in vitro transcripts of late genes *ltuA*, *hctA*, and *ltuB* were generated in the presence ($+\sigma^{66}$) and the absence ($-\sigma^{66}$) of recombinant chlamydial σ^{66} and fractionated on a DNA sequencing gel. Arrows point to the tandem transcripts generated from the cloned promoter regions. The lengths of the transcripts, estimated from the DNA sequencing ladder shown on the left, were within the range of the predicted values of 144, 165, and 125 bases for *ltuB*, *hctA*, and *ltuA*, respectively.

DISCUSSION

We used RNA probes generated by incubating *C. trachomatis* organisms isolated from host cells at 16 and 30 h p.i. with [α-^{32}P]GTP to screen genomic libraries for late-stage-specific genes. A number of potential late clones that were identified possessed insert DNA that failed to react on Northern blots of RNA extracted from chlamydia-infected host cells. These nonreacting clones may encode late genes which are transcribed at a low level or which produce highly unstable transcripts. While these genes may be of great interest, characterization of them is extraordinarily difficult and was not pursued. Several clones did react on Northern blots with RNA from infected cells, including clones encoding all or parts of *omp2* and *omp3* which had been previously cloned by other methods (1, 2, 25). In

addition, two novel genes, designated *ltuA* and *ltuB*, were identified.

ltuA encodes a transcript of about 250 bases and a short ORF of 47 codons. At present, it is not known if this short ORF is translated in vivo, and its predicted amino acid sequence bears no resemblance to known proteins. The function of *ltuA*, therefore, cannot even be speculated upon. Interestingly, *ltuA* lies downstream of a gene fragment which may encode phosphoglucoisomerase, a protein that is likely to be made during the growth phases of chlamydiae rather than in the late stage. Phosphoglucoisomerase activity has been demonstrated in cell extracts of chlamydiae (32), and the enzyme probably functions in the provision of NADH and five-carbon sugars (29, 30).

The *ltuB* gene encodes a 550-nucleotide primary transcript (transcript T1) with unusual stability properties. The 5′ end appears to break down, while the 3′ end accumulates as a stable fragment of about 240 bases (transcript T2). The significance of the breakdown and/or processing of T1 is not known. Dual transcripts are also generated from the MOMP gene and the CRP operon. In the case of the MOMP gene, two transcripts, both of which encode the structural gene, may be generated either by tandem promoters or by processing of a longer primary transcript (11, 38). In the case of the CRP operon, the two transcripts appear to be generated from a common promoter, with termination occurring frequently but not always in the intergenic region between *omp3* and *omp2* (25).

The function of *ltuB*, like that of *ltuA*, is not known. Two ORFs with no homology to known proteins start within T1. The first ORF is encoded entirely within the unstable 5′ end of T1, while the second, encoding a potential peptide of 107 amino acids, is initiated from GTG near the 5′ end of T2. This peptide would require an mRNA of at least 321 bases and is too large to be encoded in the 240-base T2 transcript. The 107-codon ORF may be adventitious, or the 3′ end of the encoding transcript may be unstable. It is also possible that none of the *ltuB* ORFs are translated and that *ltuB* RNA is in some way directly functional in the late stage of the chlamydial developmental cycle, perhaps as antisense RNA. While the function of *ltuB* remains unknown, the steady-state level of the T2 transcript in late-stage chlamydiae is high, being at least equivalent to that of the transcript encoding the small CRP (Fig. 1).

A primary aim of this study was to determine the 5′ start sites of all late genes in order to identify a possible late-stage promoter consensus or other signature sequences of late genes. It is interesting that every late gene we investigated possesses a potential −10 hexamer which matches the *E. coli* consensus in at least four positions. Indeed, the late gene consensus appears to fit the *E. coli* consensus slightly better than it fits chlamydial genes expressed during the early and midcycle stages of the developmental cycle. Many chlamydial genes analyzed thus far appear to lack a −35 hexamer which strongly resembles the *E. coli* consensus. In *E. coli*, genes which are recognized by the major sigma factor but lack a strong −35 hexamer often require class II transcription activators for activity (8). A similar requirement may account for the variance from the consensus in chlamydial genes. No convincing signature of late-stage expression could be detected between bp −90 and +25 of the late genes analyzed. The lack of any consensus may be a function of the small number of genes analyzed to date. However, Northern blot analysis of these genes indicates that not all of them are expressed at exactly the same times p.i.; thus, each may be regulated by separate repressors or other transcription factors. A sequence consisting of TNGTT was noted within the promoter regions of all late genes, often close to the −35 region. Similar sequences were lacking in the upstream regions of nonlate genes, with the exception of the *spc* operon. The significance of the TNGTT box, if any, is not known and can be determined only by analysis of appropriate mutant templates.

Although the upstream sequences of late genes suggest that transcription of these genes is initiated by σ^{66}, the actual role of any given sigma factor in the expression of chlamydial genes can be demonstrated only by functional assay. We have developed an in vitro transcription system for this purpose. The only chlamydial sigma factor identified to date is σ^{66}; consequently, it is the only one that can be tested in vitro. Using the in vitro system, we previously demonstrated that the CRP operon, the P2 promoter of *omp1*, and the antisense transcripts of the chlamydial plasmid are dependent on σ^{66} for expression (11, 12, 28). In the present study, we demonstrated that the promoter regions of late genes *ltuA*, *ltuB*, and *hctA* are also recognized by σ^{66}. We were unable to detect *hctB* in vitro transcripts directly on sequencing gels, although we were able to detect transcripts from templates encoding this gene indirectly by primer extension. It is possible that *hctB* is poorly expressed in vitro and that the primer extension assay is more sensitive than the direct assay in detecting transcripts present in low copy number. Our detection of transcripts by primer extension and the apparent monocistronic nature of *hctB* suggest that the in vitro templates assayed do contain the *hctB* promoter. Unfortunately, our primer extension assay is not quantitative; therefore, we were not able to determine the sigma factor requirement for *hctB* expression.

In summary, the present study and a previous study (28) suggest that the CRP operon, *hctA*, *ltuA*, and *ltuB* require the major chlamydial sigma factor for gene expression. A role for σ^{66} in the expression of *hctB* was not demonstrated but cannot be excluded by our studies. It seems likely, therefore, that a specific late-stage sigma factor is not required for at least some gene expression late in the chlamydial developmental cycle.

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