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SP01 Gene 27 Is Required for Viral Late Transcription

JONATHAN R. GREENE,* BARRY K. CHELM, AND E. PETER GEIDUSCHEK
Department of Biology, University of California at San Diego, La Jolla, California 92093

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The SP01 mutant *sus* HA20 (gene 27) was found to be defective for synthesis of viral late RNA. It is known that gene 27 is also required for viral DNA replication. The SP01 gene 27 product resembles the T4 gene 45 product, which also has a dual role in viral DNA replication and late transcription.

Infection of *Bacillus subtilis* with phage SP01 generates a well-ordered temporal sequence of viral gene expression (10). Biochemical studies on phage regulatory mutants that are defective in various stages of the transcription program (9) have shown that the corresponding genes code for proteins which bind to the host's RNA polymerase core (7, 8, 26) to direct the recognition of novel promoters (6, 25). Talkington and Pero (24) used Southern hybridization (23) to map physically the various temporal classes (10) of SP01 RNA to *Eco*RI fragments of SP01 DNA. We adopted the same analysis to show that the SP01 mutant *sus* HA20 (gene 27), which is known to be defective for viral DNA synthesis (15), is also defective for late transcription. By comparing *sus* HA20 with mutants in SP01 genes 33 and 34, which are known to be defective for viral late transcription but replicate viral DNA normally (9, 15), we found that the defects of their respective viral transcription programs are not entirely identical.

Figure 1 shows the Southern hybridization patterns of RNA pulse-labeled at various times after infection of *B. subtilis* 168M (Su^-) with wild-type SP01 phage. The *Eco*RI fragments of SP01 are numbered in Fig. 1 according to the map derived by Pero et al. (Fig. 2; 16). Given the coarse level of resolution provided by the *Eco*RI digest, it was nevertheless possible to assign *Eco*RI fragments to different temporal classes of the transcription program. In this respect, the data in Fig. 1 (schematically represented in Fig. 2) are consistent with previous in vivo and in vitro studies (1, 4, 16, 20, 24, 25), which have been reviewed (11).

RNA was pulse-labeled late after infection with wild-type or *sus* HA20 (gene 27) mutant SP01 phage and hybridized to excess SP01 DNA in solution as previously described (10). It was found that 12.1% of the radioactivity present in RNA labeled 23 to 26 min after wild-type infection was hybridized, whereas only 0.6% of the radioactivity present in RNA similarly labeled after *sus* HA20 infection was hybridized (data not shown). This is consistent with previous work (9, 21; M. C. Añon, Ph.D thesis, Universidad Nacional de la Plata, La Plata, Argentina, 1974) and our own current results (data not shown), which indicate that SP01 replication-defective (DO) mutants produce greatly reduced proportions of viral RNA at late times of infection, i.e., long after the normal onset of viral DNA synthesis. (Phage DNA synthesis was found to begin between 12 and 14 min after wild-type infection (data not shown) under the conditions described in the legend to Fig. 1.) It should be noted that the percentage of label in wild-type RNA hybridizing to SP01 DNA was lower than seen previously (10), probably due to different growth conditions. The hybridization patterns of late *sus* HA20 RNA and late wild-type SP01 RNA (Fig. 3) were strikingly different. *Sus* HA20 RNA failed to hybridize to DNA fragments, such as *Eco*RI 2, 8, 16, 17, or 20, which contain late genes. Wild-type and *sus* HA20 RNA pulse-labeled before the normal onset of phage DNA synthesis were qualitatively similar (data not shown). However, fragments which were first transcribed at a low level several minutes after infection and heavily transcribed only after the normal onset of phage DNA synthesis, such as *Eco*RI 12, 19, and 25, were not detectably transcribed at late times after infection with *sus* HA20.

To further characterize the phenotype of *sus* HA20, we compared it with mutants in either or both genes 33 (*sus* F14) and 34 (*sus* F4). It is clear (Fig. 4) that phage transcription late after infection with *sus* HA20 resembles that of *sus* F4, *sus* F14, and the double mutant *sus* F4-F14 more than that of wild-type SP01, confirming that *sus* HA20 is also defective for late transcription. However, there were certain significant differences. *Eco*RI 12 and 21 were more heavily transcribed late after infection with *sus* F4, *sus* F14, or *sus* F4-F14 than with *sus* HA20. On the other hand, *Eco*RI 13, 14, and 15 were more heavily transcribed late after infection with *sus* HA20 than with *sus* F4, *sus* F14, or *sus* F4-F14.

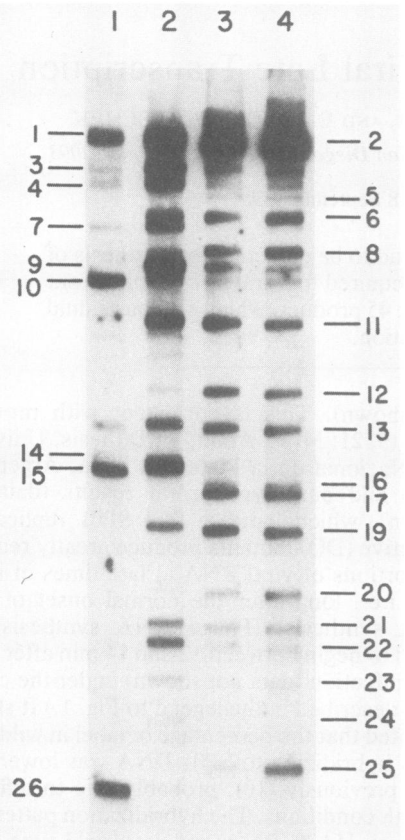


FIG. 1. Time sequence of phage SP01 wild-type transcription. RNA was pulse-labeled at various times after infection of *B. subtilis* 168M with wild-type SP01, isolated, and hybridized to nitrocellulose-bound *Eco*RI fragments of SP01 DNA. The times of labeling were: lane 1, 0.5 to 3 min postinfection (p.i.); lane 2, 7 to 10 min p.i.; lane 3, 16 to 19 min p.i.; lane 4, 25 to 28 min p.i. Phage DNA was purified from CsCl-purified phage as described previously (20) and cleaved with *Eco*RI endonuclease (the generous gift of T. Elliott) under *Eco*RI* conditions (17) in the presence of 16% glycerol. Approximately 25 (hour) U/μg of DNA were required to produce the digestion pattern presented here. Restriction fragments were separated on preparative 1.0 or 1.2% agarose gels in E buffer as described (2). After staining with ethidium bromide and photography, the gels were prepared for the transfer of DNA to nitrocellulose paper (Millipore Corp., Bedford, Mass.) as described by Wahl et al. (27) to ensure quantitative transfer of high-molecular-weight DNA fragments. *Eco*RI fragments of SP01 DNA were labeled at their 5′ ends with T4 polynucleotide kinase (the generous gift of S. Brennan) and [γ-^{32}P]ATP (Amersham Corp., Arlington Heights, Ill.) according to Maxam and Gilbert (14). Labeled DNA was electrophoresed and transferred to nitrocellulose in parallel with unlabeled DNA to monitor the extent of transfer of DNA fragments. After transfer was complete (16 to 20 h were allowed), the filters were sliced so as to contain approximately 2 μg of SP01 DNA on each strip, dried at 80°C in vacuo for 4 h, and stored under vacuum at room temperature until use. Overnight cultures of *B. subtilis* 168M were grown in dephosphorylated B medium (9) supplemented with 10^{-4} M NaH_2PO_4, diluted 100-fold into B medium supplemented with 10^{-5} M NaH_2PO_4, and grown at 37°C. At an absorbance at 500 nm of 0.3 (6×10^7 cells per ml), 25 ml of cells was infected with five phage per bacterium. At the indicated times p.i. 2 ml of infected cells was transferred to a clean scintillation vial and incubated with shaking at 37°C for 2.5 or 3 min in the presence of 500 μCi of carrier-free $H_3{}^{32}PO_4$ (ICN Pharmaceuticals, Inc., Irvine, Calif.). Labeling was stopped by adding the cells to 5 ml of ice-cold Spizizen salts (10), which also contained 0.01 M NaN_3 and approximately 3×10^{10} unlabeled and uninfected *B. subtilis* 168M "carrier" cells. The latter were prepared by growing to an absorbance at 500 nm of 1.0 (2×10^8 cells per ml), collecting by centrifugation at 6,000 × *g* for 5 min, and suspending in a 1/30 volume of Spizizen salts containing 0.01 M NaN_3. Carrier-supplemented labeled cells were collected by centrifugation at 6,000 × *g* for 5 min and suspended in 0.6 ml of Spizizen salts containing 0.01 M NaN_3. Sucrose (Schwartz/Mann, Orangeburg, N.Y.), lysozyme (Sigma Chemical Co., St. Louis, Mo.), and chloramphenicol (Sigma) were added to final concentrations of 16%, 500 μg/ml, and 100 μg/ml, respectively, and the cell suspension was incubated at 37°C for 2 min. Lysis was completed by adding 1.5 ml of hot (100°C) 0.01 M Tris-chloride (pH 7.5)–0.1 M NaCl–0.05 M EDTA–6% Sarkosyl–1.2 mM aurin tricarboxylic acid followed by incubation at 100°C for 5 min. Solid CsCl (1.2 g) was immediately added for each ml of lysate. The resulting solution was cooled and layered over a 1.5-ml cushion of 5.6 M CsCl in 0.1 M Tris-chloride (pH 7.5)–0.05 M EDTA. After balancing tubes with 4% Sarkosyl in 0.1 M Tris-chloride (pH 7.5), the RNA was pelleted in a Spinco SW50.1 rotor for 19 to 22 h at 35,000 rpm and 20°C (3), suspended in 0.01 M Tris-chloride (pH 7.5)–0.01 M NaCl–0.001 M EDTA–0.1% sodium dodecyl sulfate and extracted twice with a 24:1 (vol/vol) mixture of chloroform and isoamyl alcohol. Some RNA preparations were then heated to 100°C for 2 min, quickly chilled, and filtered through buffer-equilibrated nitrocellulose membranes (Millipore). RNA was precipitated with ethanol after addition of LiCl to 0.5 M, suspended in 2× SSC (0.15 M NaCl plus 0.015 M sodium citrate), and stored at −20°C. The labeled RNA ranged in size from 0.2 to 1.5 kilobases. [^{32}P]RNA was hybridized to nitrocellulose-bound DNA in 50% formamide–4× SSC–0.1% sodium dodecyl sulfate in sealed plastic bags, overnight at 37°C, as described elsewhere (1). Nitrocellulose strips were then washed twice in 50% formamide–4× SSC and twice in 2× SSC (20 min per wash at room temperature), dried, and exposed to DuPont Cronex IV film next to a DuPont QIII intensifying screen at −70°C. Most films were flashed before exposure (13). In the experiment shown here, 6.4×10^6 cell equivalents of total [^{32}P]RNA (approximately 0.5 μg) were allowed to hybridize to each strip. The input radioactivity for each lane was as follows: lane 1, 2.2×10^5 cpm; lane 2, 1.3×10^5 cpm; lane 3, 1.2×10^5 cpm; and lane 4, 2.8×10^5 cpm. The exposure time for autoradiography was 119 h.

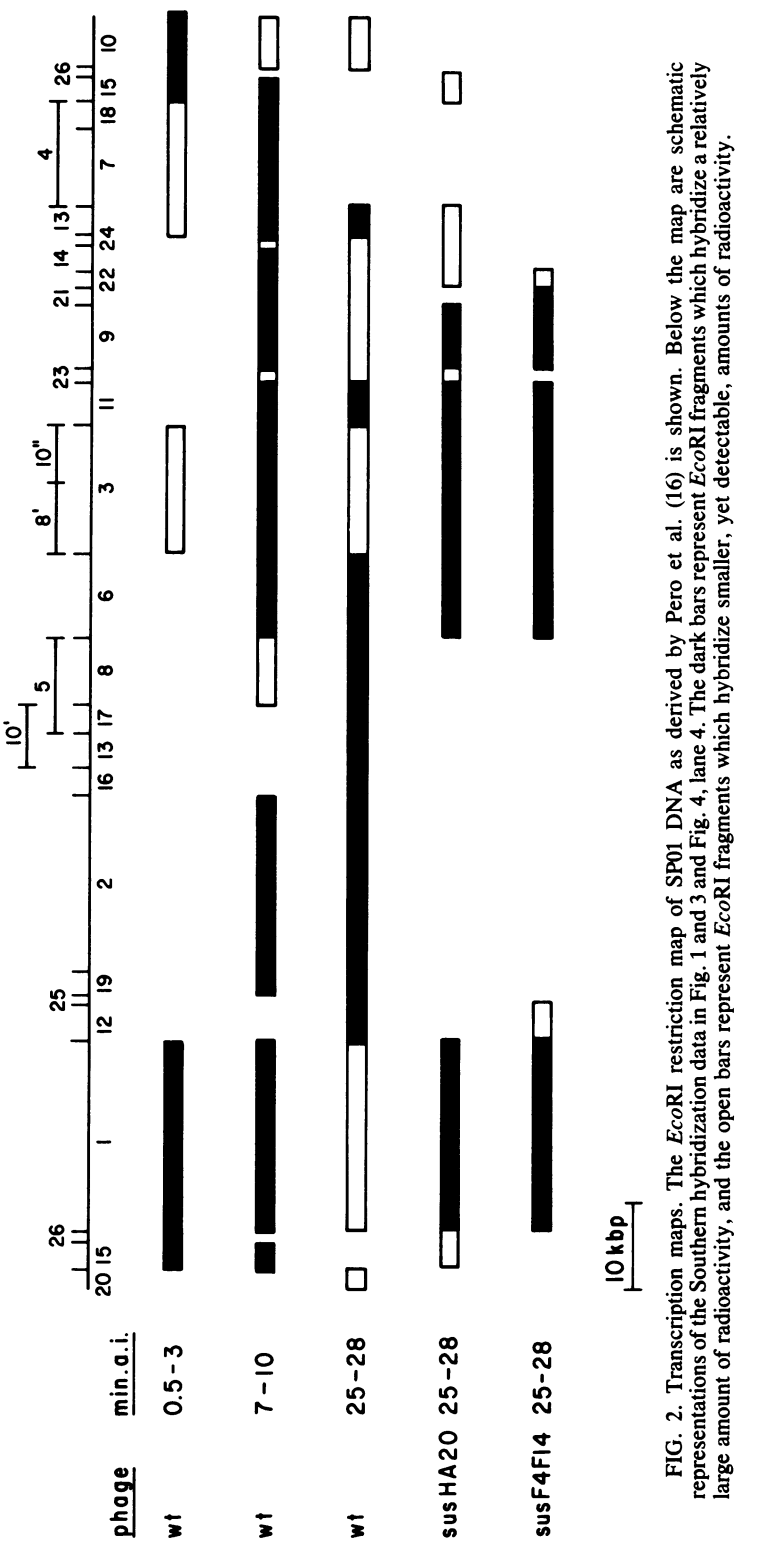


FIG. 2. Transcription maps. The *Eco*RI restriction map of SP01 DNA as derived by Pero et al. (16) is shown. Below the map are schematic representations of the Southern hybridization data in Fig. 1 and 3 and Fig. 4, lane 4. The dark bars represent *Eco*RI fragments which hybridize a relatively large amount of radioactivity, and the open bars represent *Eco*RI fragments which hybridize smaller, yet detectable, amounts of radioactivity.

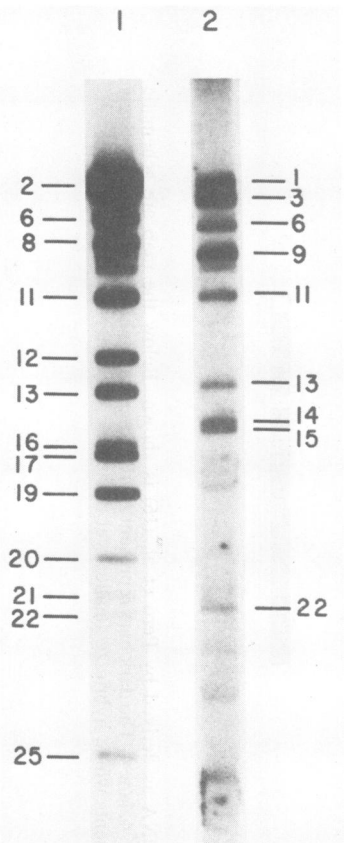


FIG. 3. Transcription defect of mutant *sus* HA20. RNA labeled 25 to 28 min p.i. with wild-type SP01 (lane 1) or *sus* HA20 (lane 2) was hybridized to nitrocellulose-bound *Eco*RI fragments of SP01 DNA as described in the legend to Fig. 1. The cell equivalents of RNA, input radioactivity, and the exposure time for autoradiography were: lane 1, 2.4×10^6 cell equivalents, 2.8×10^5 cpm, 70 h; lane 2, 2.4×10^6 cell equivalents, 1.4×10^5 cpm, 382 h.

Relative to wild-type SP01, *sus* HA20 and *sus* F4 infections involved somewhat slower repression of certain phage early genes (data not shown; cf. reference 24). For example, late RNA from either *sus* HA20- or *sus* F4-infected cells hybridized weakly to *Eco*RI 26. There were also minor differences between the RNA synthesized late after infection with *sus* F4 and with *sus* F14 or *sus* F4-F14 (Fig. 4, lanes 2, 3, and 4) which are reasonably consistent with previous work (24). Neither of these last two subtleties has been analyzed in detail by us. Our interest has centered on the comparison of late transcription by *sus* F4, *sus* F14, and *sus* HA20.

We also compared phage RNA synthesis during infection with *sus* HA20 and two other DO mutants of SP01 (Fig. 5). The latter were chosen to represent each of the two SP01 replication gene clusters, genes 21 to 23 (*sus* F2, gene 21) and genes 27 to 32 (*sus* F38, gene 32) (15). In agreement with previous experiments with the SP01 DO mutant *sus* F30 (gene 22) (24), it is apparent in Fig. 5 that late RNA from cells infected with *sus* F2 or *sus* F38 hybridizes to restriction fragments, such as *Eco*RI 2, 8, 16, 17, and 19, which contain late genes. RNA-DNA hybridization competition experiments (Añon, Ph.D. thesis) suggest that mutants within each of the two SP01 DO gene clusters have similar phenotypes with regard to late transcription. This observation leads us to conclude that *sus* HA20 is different from the other SP01 DO mutants in its inability to synthesize viral late RNA.

We have shown that SP01 gene 27 is required for the synthesis of viral late RNA. Heintz and Shub (N. Heintz, and D. A. Shub, submitted for publication) have independently found that *sus* HA20 is unique among SP01 DO mutants in being globally defective for the synthesis of viral late proteins. The involvement of gene 27 protein is evidently at the level of transcription. Although the relationship between viral DNA replication and late transcription during SP01 infection is not entirely resolved, gene 27 is now known to be required for both. In this respect the SP01 gene 27 product is very similar to the T4 gene 45 protein (28), which is part of the viral DNA replication enzyme complex (22) and also interacts with the late transcription complex (5, 18). In that connection, an interesting temperature-sensitive mutant in gene 27, which appears to separate the replication and late transcription-defective phenotypes, has been reported (12).

Reports of transcription of SP01 late RNA in vitro have stressed the competence of RNA polymerase containing SP01 gene 33 and 34 products for this task, yet selective late transcription in vitro (26) also requires the host's δ protein, possibly to prevent indiscriminate template binding, and antibody against the host's σ subunit, presumably to suppress early transcription. This in vitro system may lack components which are required in vivo; the SP01 gene 27 product might be one of the missing elements.

RNA labeled late after infection with *sus* HA20 is slightly different from RNA labeled late after infection with *sus* F4, *sus* F14, or *sus* F4-F14 (Fig. 4). RNA labeled before the onset of DNA replication after infection with either of these mutants was qualitatively similar to that of wild type (data not shown). Whether the differences in late transcription are the result of gene dosage (due to the replication defect of *sus* HA20) or reflect different roles for the gene 27, 33, and 34 products in the negative regulation of viral middle gene sequences requires further analysis. It is appropriate to point out that the

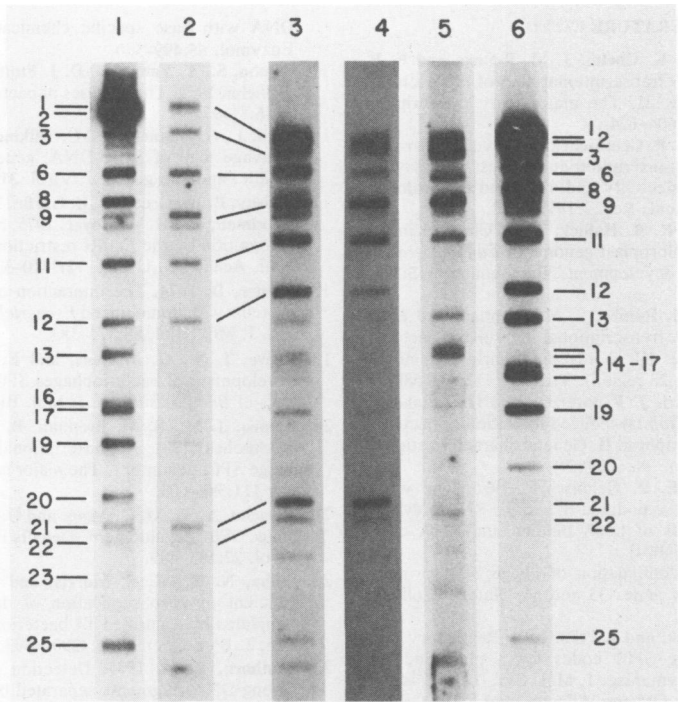
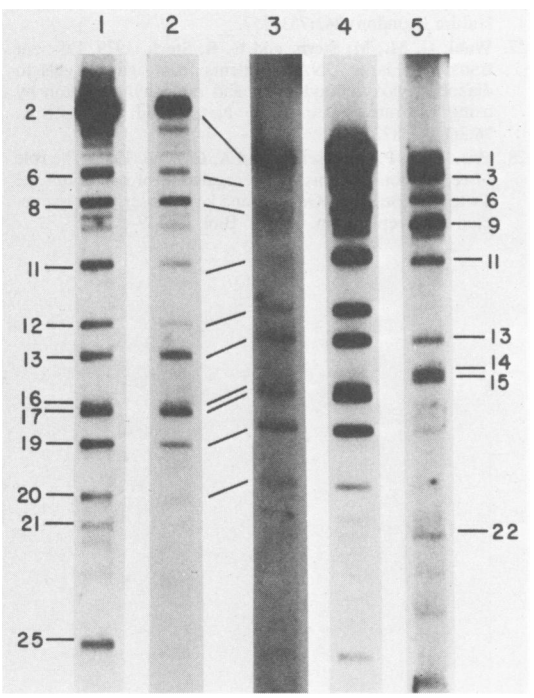


FIG. 4. Comparison of late-transcription-defective mutants. RNA labeled at the indicated late times p.i. with wild-type SP01 or with the various late transcription-defective mutants of SP01 was hybridized to nitrocellulose-bound *Eco*RI fragments of SP01 DNA. Cell equivalents, input radioactivity, and exposure times for autoradiography were as follows: lane 1, wild-type SP01, 25 to 28 min p.i. (as in Fig. 1, lane 4); lane 2, *sus* F14, 22 to 25 min p.i., 4.0×10^7 cell equivalents, 7.3×10^4 cpm, 193 h; lane 3, *sus* F4, 22 to 25 min p.i., 4.8×10^7 cell equivalents, 6.0×10^4 cpm, 170 h; lane 4, *sus* F4-F14, 25 to 28 min p.i., 4.0×10^6 cell equivalents, 2.8×10^4 cpm, 118 h; lane 5, *sus* HA20, 25 to 28 min p.i. (as in Fig. 3, lane 2); lane 6, wild-type SP01, 25 to 28 min p.i. (as in Fig. 3, lane 1).



gene 27 product appears to be an early protein (4, 19; Heintz and Shub, submitted for publication).

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FIG. 5. Comparison of DO mutants. RNA labeled at the indicated late times after infection with wild-type SP01 or with various DO mutants of SP01 was hybridized to nitrocellulose-bound *Eco*RI fragments of SP01 DNA. Cell equivalents, input radioactivity, and exposure times for autoradiography were as follows: lane 1, wild-type SP01, 25 to 28 min p.i. (as in Fig. 1, lane 4); lane 2, *sus* F2, 22 to 25 min p.i., 8.0×10^7 cell equivalents, 6.7×10^4 cpm, 193 h; lane 3, *sus* F38, 22 to 25 min p.i., 3.2×10^7 cell equivalents, 1.6×10^5 cpm, 170 h; lane 4, wild-type SP01, 25 to 28 min p.i. (as in Fig. 3, lane 1); lane 5, *sus* HA20, 25 to 28 min p.i. (as in Fig. 3, lane 2).

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