Fertility inhibition gene of plasmid R100

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ABSTRACT

The finO gene of R100 was isolated from the FinO[+] transducing phage VAλ57. The limits of the gene were determined by BAL31 digestions and by analysis of deletion mutations derived from an internal restriction site.   The DNA sequence contained an open reading frame of 558 nucleotides that would encode a protein of 21,268 daltons. Synthesis of such a protein was observed only when the fragment was cloned in front of the TAC promoter. Deletions entering the large open reading frame from either end were FinO[-], while internal frame shift mutations retained high FinO activity.   One such strain had a 13 bp internal deletion that would produce a protein of 63 amino acid residues of which 21 were basic. We were consequently unable to rigorously establish that the 558 base orf encoded a finO product. The strand opposite the large open reading frame contained several transcription termination signals, and it is possible that the active gene product is one or two small RNAs from this strand.

INTRODUCTION

Transcription of the 33 kb traYZ transfer operon of the sex factor F and related plasmids requires the product of the traJ gene (1) which maps immediately upstream of the traYZ operon (2). The traJ gene encodes a cytoplasmic protein of 27,000 daltons that is present in approximately 4000 copies per cell (3). Expression of the traJ gene is negatively regulated by two trans-acting genes, finP, which is plasmid specific, and finO (4), which is not plasmid specific. The latter is the gene supplied by the plasmid R100 to inhibit the transfer of Hfrs in the original fi[+] experiments of Egawa and Hirota (5) and Watanabe et al. (6). FinO and FinP are parts of the FinO/P model that attempts to explain fertility inhibition (4). Briefly, this model refers to the positive regulation of traYZ transcription by TraJ protein and the double negative regulation of traJ expression by finO and finP. Recent data have suggested that traJ expression may be translationally regulated by finO as well as by the cpx and dye (sfr) genes of the bacterial chromosome (3), but a new model has not been proposed.

The finP genes of the sex factor F (7), R100 (8), and several related

plasmids (9) have been identified and sequenced. All map inside their respective traJ genes and apparently encode small RNA molecules that are anti-sense to the ribosome binding sites of traJ mRNA. This suggests that the finP portion of the FinO/P control might be similar to translational control mechanisms that limit replication of the plasmids R1 and R100 (10,11). The finO gene of R100, located downstream of the traYZ operon (12), has been cloned (12) and mutated (13). It was reported that the finO gene of the related plasmid R6-5 encodes a protein of 21,000 daltons (14), but those data were not unequivocal. We report here the subcloning and sequencing of the R100 finO gene as well as our attempts to define the nature of the finO product.

MATERIALS AND METHODS
Bacterial strains, plasmids, phage, media

E. coli strain HB101 (F⁻,λ⁻,hsdS20 (r$_B$⁻ m$_B$⁻) recA13, lacY1, proA2, leu, ara-14, galK2, rpsL20, xyl-5, mtl-1 supE44) was obtained from D. Miller and used as host for all pWD plasmids. Its F'lac derivative, VA8100, made from a cross with ED395 (F'lac) (15) was used as host for tests of finO. CSR603 (recA1, uvrA6, phr-1) was obtained from W.D. Rupp. JC3272 (his, trp, lys, str, gal, lac, λ^R (λdef)) was used as recipient in quantitative crosses. JM105 (Δlac-pro, thi, strA, endA, sbcB15, hsdR4 F'traD36, proAB, lacM15, lacI^q) was obtained from Pharmacia PL Biochemicals and used as host for pKK223. The plasmid used as cloning vehicle for isolation and growth of fragments was pHP34 (obtained from H.M. Krisch) which is identical with pBR322 except that it has a 10 bp insert in the EcoRI site that contains a SmaI site. This allows blunt-end cloning into the SmaI site and removal of the cloned fragment with EcoRI to regenerate EcoRI-cleaved pBR322 (16). The TAC promoter vector pKK223 was obtained from Pharmacia PL Biochemicals and used according to directions supplied by them. Similar plasmids were originally described by Amann et al. (17). Bacteriophages f1, µ2, and VAλ57 have been described (12,15). L broth and ML agar were described previously (18).
Enzymes

Calf intestinal phosphatase and bacterial alkaline phosphatase were from P-L Biochemicals, Inc.; Klenow fragment, DNA polymerase I, and deoxyribonuclease I were from Bethesda Research Laboratories, Inc.; restriction endonucleases, T4 polynucleotide kinase, T4 DNA ligase and BAL31 exonuclease were from New England BioLabs, Inc. Restriction enzymes were used as described by the supplier. BAL31 digestions were performed as described by Maniatis et al. (19).

Cloning

The cloning vehicle for this work was SmaI-digested pHP34. Before use it was dephosphorylated with calf intestinal phosphatase according to Maniatis et al. (19). The DdeI and HinfI fragments shown in Fig. 1 were first filled by using DNA polymerase Klenow fragment and the four deoxynucleotide triphosphates under conditions described by Maniatis et al. (19). CaCl$_2$ transformation was performed as described by Maniatis et al. (19). Transformation mixtures were allowed to grow 1 h in L broth with glucose at 0.2% before plating on ML agar plates containing either ampicillin (100 μg/ml) or tetracycline (10 μg/ml). FinO$^+$ fragments were identified by the μ2 Giemsa test (12), and likely candidates were then screened for EcoRI fragments of the predicted sizes. Identity of the fragments was established by digestion of the plasmids with other restriction enzymes.

FinO test

Quantitative analysis of FinO was measured in standard 37°C, 30 min matings (12,20) as the amount of F'lac transferred into JC3272 from VA8100 strains carrying the recombinant plasmid being tested. This donor ability from stable heterozygous donors is expressed as transconjugants per 100 donor cells. Values equal to or less than 10^{-5} indicate no detectable transfer. A value of 100 indicates uninhibited transfer. A single copy of finO reduces transfer of F or R100-1 from 100- to 1000-fold depending upon the bacterial strain used.

Preparation of plasmid DNA and isolation of fragments

Small quantities of plasmid DNA were made from 1-2 ml of shaken overnight L broth cultures by the Holmes and Quigley method (21). Initially, large scale plasmid preparation was by CsCl equilibrium centrifugation following amplification, cell lysis, and DNA precipitation as described by Humphreys et al. (22). Later preparations were purified by chromatography on NACS37 (23). The desired DNA restriction fragments were isolated from horizontal Tris-borate agarose gels by electroelution into DEAE paper and then by extraction from the paper using a modification of the method described by Dretzen et al. (24). DNA was eluted from the paper with the high salt buffer described (20 mM Tris-Cl, pH 7.8, 1 mM EDTA, 1.5 M NACl), using three consecutive 37°C incubations of the paper with 150 μl salt in a 500 μl Eppendorf tube. Each eluate was removed by punching a hole in the Eppendorf top and bottom, placing it in a 1.5 ml Eppendorf tube and centrifuging for 10 min in a microfuge. Eluates were pooled and the DNA recovered by ethanol precipitation. This

method was used for all sizes of DEAE paper up to 10 cm^2 and gave yields of >80%.

DNA sequencing procedures

Labeling of 5' ends following bacterial alkaline phosphatase treatment was done with [γ-^{32}P]ATP and T4 polynucleotide kinase as described by Maxam and Gilbert (25). Labeling of 3' ends was done by adding 2 units of E. coli DNA polymerase I to a 50 μl volume containing 67 mM Tris-Cl, pH 7.4, 67 mM NaCl, 7 mM MgCl$_2$, at least 10 pM DNA fragment, and 50-100 μCi of the appropriate [α-^{32}P]deoxynucleotide and incubating for 15 min on ice (D. Miller, personal communication). Secondary restriction enzyme cleavages were made where necessary and single end-labeled fragments were isolated from agarose gels as described above. The end-labeled fragments were sequenced using the chemical degradation method of Maxam and Gilbert (25,26) and the products separated on 20% and 6% polyacrylamide, 7 M urea gels. Sequence data were analyzed using the Beckman Microgenie program developed by Queen and Korn (27).

Isolation of pKK223 derivatives

Ligation of EcoRI fragments into the EcoRI site of pKK223, and transformation into JM105 selecting for ampicillin resistance was accomplished by standard procedures (19). Many transformants were detected simply by standard screening procedures based upon plasmid size (21). Other finO transformants were detected by patching transformants onto ML plates, allowing the patches to grow 8-16 h, and then inoculating by the replica plate technique both a minimal glucose plate to test for the F'pro plasmid in JM105 and a Giemsa plate spread with 10^{11} f1 phage to test for finO activity (12). Even though the F'pro plasmid carries a traD mutation, f1 phage plate readily on these mutants unless expression of the transfer operon is repressed by finO. Transformants that grew on both plates were streaked and mini-lysates (21) examined for plasmid. This phenotype proved unstable; some isolates appeared to lose the F'pro at a very high rate, and others maintained both plasmids but lost finO activity. Cultures that retained the unstable parental phenotype were detected throughout purification by the replica plate test. These were frozen at -70°C in 50% glycerol upon isolation. Frozen cultures were used as the inocula for overnight cultures for protein synthesis. The presence of finO in these purified cultures was tested by introducing the finO mutant R100-1 and looking for reduced transfer of R100-1.

Protein synthesis by the TAC promoter of pKK223

Cultures of JM105 containing the pKK233 derivatives were grown to a density of 1x10^8 cells per ml in L-broth at 37°C. These cultures were inoculated

from fresh overnight standing cultures in the same medium. The exponential cultures (0.5 ml) were diluted into 9.5 ml prewarmed identical medium containing IPTG at a final concentration of 1×10^{-4} M and shaken for 90 min. One ml samples were spun 3 min in an Eppendorf microfuge, washed in 1 ml sterile 0.01 M Tris pH 7.2, and suspended in 0.09 ml lysis buffer (28). The mixtures were allowed to sit at room temperature for 1 h, and 0.01 ml of 0.1% bromophenol blue was added. After heating at 100°C for 2 min, 0.015 ml samples were loaded onto 15% polyacrylamide gels that were prepared as described by Bethesda Research Labs (Focus 6:3). Gels were run at 5 V/cm, stained with Coomassie Brilliant Blue (0.25%) in methanol:acetic acid:water = 5:5:1, and destained in 10% methanol in the presence of excess Dowex-1 beads. Prestained MW standards from Bethesda Research Labs were used as markers.

RESULTS
Mapping of finO

The FinO transducing phage VAλ57 (12) was used as the primary source of R100 finO DNA. We knew from its structure, the sequence of lambda (29), the restriction map of the related plasmid R6-5 (14), and the map of the region of NR1 (R100) (30,31), that a PstI digest of VAλ57 would contain finO on a fragment of about 4.7 kb. This PstI fragment from VAλ57 was cloned into the PstI site of pBR322 to form the plasmid pWD11 (Dempsey, manuscript submitted). All of the finO activity of pWD11 was contained on a 1130 bp HinfI R100 fragment. This fragment was isolated, filled, and ligated into SmaI-digested pHP34 producing not only the desired HinfI fragment-containing plasmid (pWD47), but three plasmids containing partial HinfI fragments in which one end of the parental fragment was lost. These plasmids (pWD49, pWD50, and pWD51) appeared to be formed by normal blunt-ended ligation between one end of the HinfI fragment and one end of pHP34 and by "abnormal" ligation of the other end of pHP34 to the middle of the HinfI fragment. The structures of the plasmids as determined by analysis of restriction enzyme digests together with their finO activities are shown in Fig. 1. pWD49 contained 750 bp of the "right" end of the fragment, pWD50 contained 685 bp of the "left" end and pWD51 contained 712 bp (by sequencing) of the "right" end of the HinfI fragment. Fig. 1 shows these four plasmids and a fifth derivative, pWD44 that contained the central DdeI fragment from the HinfI piece. It lacked FinO activity. Each of these fragments was cloned in both orientations either in the original isolation or following EcoRI digestion and religation. Derivatives of each fragment in either orientation produced finO activities essen-
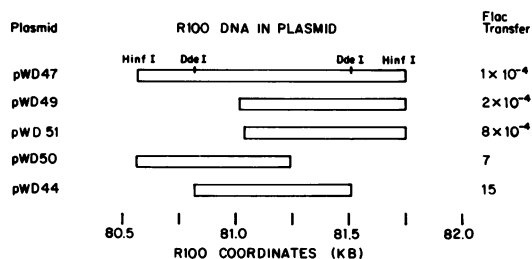
Figure 1. Restriction fragments used in defining limits of the finO gene. Each fragment was cloned as a blunt-ended piece into the SmaI site of pHP34 to form the plasmids named on the left. The ability of the plasmids to inhibit transfer of F'lac from a stable heterozygote is shown on the right as F'lac transconjugants per 100 donor cells. The value for no FinO activity in this particular strain (HB101) was 15 and for no transfer (FinO+) was < 10^{-5}.

tially the same as the values shown in Fig. 1. The religated derivatives of pWD51, which contained the shortest FinO+ fragment, were pWD64 (opposite orientation) and pWD65 (original orientation). These plasmids were used to generate the BAL31 deletions described below. The EcoRI fragment from pWD51 was cloned also into the EcoRI site of pBR325 in both orientations. These derivatives, as well as pWD64 and pWD65 had identical and full finO activity (data not shown).

Protein synthesis

We were unsuccessful at demonstrating any unique protein product from any of the plasmids shown in Fig. 1 except when we cloned them in front of the strong TAC promoter in PKK223 (see below). Using SDS-polyacrylamide gels, we analyzed extracts of plasmid-bearing strains of HB101 (Coomassie Blue staining) and the maxicell strain CSR603 (^{35}S methionine labeling) without finding any trace of a unique protein. For both simple extracts and maxicell labeling we tried all published significant variations without success. Our negative results were consistent with the observation of Cheah et al. (32) using maxicell analysis of the R6-5 finO gene.

The R100 fragments from pWD47, pWD50, and pWD51 were isolated from EcoRI digests and cloned into the EcoRI site of the TAC promoter plasmid pKK223 in both orientations for further protein testing. Only the fragment from pWD51 made a detectable new protein on induction with IPTG. Using this result, we determined that the effective concentration for IPTG appeared to plateau between 1×10^{-5} M and 5×10^{-4} M (data not shown). At 10^{-3} M significantly less induced protein was seen. A similar exploration of the effect of time on the amount of protein seen showed that the maximum protein was seen between 1 and
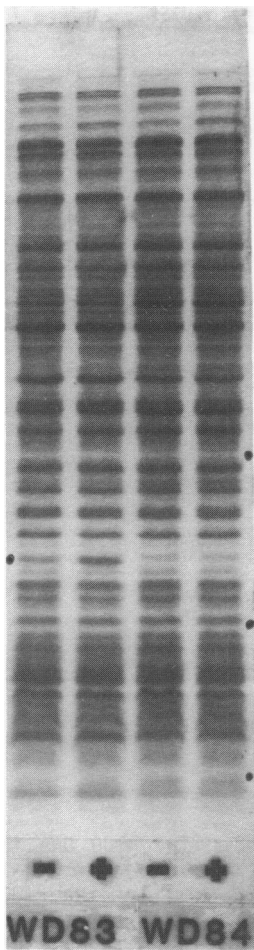
Figure 2. Photograph of Coomassie-Blue stained proteins on a 15% polyacrylamide-SDS gel. pWD83 and pWD84 are pKK223 derivatives containing the R100 fragment from pWD51 immediately downstream from the TAC promoter. In pWD83 the HinfI end of the fragment is distal to the TAC promoter, and in pWD84 it is proximal. The "+" and "-" signs indicate the presence and absence of the TAC inducer isopropyl-thiogalactoside. The dots on right represent the positions of stained standards at 25.7, 18.4, and 6.2 K daltons. The dot on left indicates the position of the induced protein at 20.6 K daltons.

2 h after exposure to IPTG (data not shown). Only trace amounts of protein were detectable at 3 h. These results are quite different from those seen with the lambda repressor in which the amount of repressor increased for hours after induction (17).

The results for the fragment from pWD51 in both orientations at maximum conditions are shown in Fig. 2. The size of the induced protein was 20,600 daltons, and it was made only when the intact HinfI site (right side of fragment as oriented in Fig. 1) was distal to the TAC promoter. This protein testing system depends upon the presence of the lacI^q gene to keep the TAC promoter turned off unless induced and this lacI^q gene is carried on an F'pro$^+$ plasmid in the testing strain. When resistance to the male specific phage f1

```
              -200
        GG AATTATACGA GGAAGCTGAC GCTGCCATTA TGATATTTAT GAAGGCGCGT TTTTTGAACG
        CC TTAATATGCT CCTTCGACTG CGACGGTAAT ACTATAAATA CTTCCGCGCA AAAAACTTGC

                                       -100
TGTGGCGGCA GTACAGACGC AATGGTTTAA AAAACATCTT TAATTGTCGG TATTAATATG TCAGAGGTCG
ACACCGCCGT CATGTCTGCG TTACCAAATT TTTTGTAGAA ATTAACAGCC ATAATTATAC AGTCTCCAGC

                                                                    -1
TTGAGGTCAG CTTAATATCA TAGTGAGTAG TTTCAACGAC CTAGAGGACA TATTTTCACA AAGGTCAGGG
AACTCCAGTC GAATTATAGT ATCACTCATC AAAGTTGCTG GATCTCCTGT ATAAAAGTGT TTCCAGTCCC

1                       RsaI
CAATCATCAC AGAGCAGAAA CGACCGGTAC TGACACTGAA ACGGAAAACG GAAGGGGAAA CACCGACCCG
GTTAGTACTG TCTCGTCTTT GCTGGCCATG ACTGTGACTT TGCCTTTTGC CTTCCCCTTT GTGGCTGGGC

GAGCCGGAAA ACCATCATCA ATGTCACCAC GCCACCAAAA TGGAAGGTGA AAAAGCAGAA ACTGGCGGAG
CTCGGCCTTT TGGTAGTAGT TACAGTGGTG CGGTGGTTTT ACCTTCCACT TTTTCGTCTT TGACCGCCTC

           XmaI                                            200
AAGGCTGCCC GGGAAGCAGA GCTGACAGCA AAAAAGCGC AGGCCAGACA GGCGCTGTCC ATTTATCTGA
TTCCGACGGG CCCTTCGTCT CGACTGTCGT TTTTTTCGCG TCCGGTCTGT CCGCGACAGG TAAATAGACT

ACCTGCCCTC GCTGGATGAG GCCGTGAACA CCCTGAAACC CTGGTGGCCG GGATTATTTG ACGGTGACAC
TGGACGGGAG CGACCTACTC CGGCACTTGT GGGACTTTGG GACCACCGGC CCTAATAAAC TGCCACTGTG

                    300
ACCCCGACTT CTGGCCTGCG GTATCCGGGA CGTGTTACTG GAAGACGTGG CGCAGCGGAA TATCCCGCTC
TGGGGCTGAA GACCGGACGC CATAGGCCCT GCACAATGAC CTTCTGCACC GCGTCGCCTT ATAGGGCGAG

                          HaeIII                    400
TCGCATAAAA AACTGCGGCAG GGCGCTGAAG GCCATCACCC GTTCAGAAAG CTATCTGTGT GCCATGAAAG
AGCGTATTTT TTGACGCGTC CCGCGACTTC CGGTAGTGGG CAAGTCTTTC GATAGACACA CGGTACTTTC

CCGGTGCCTG CCGGTATGAC ACGGAAGGGT ATGTGACGGA GCATATTTCT CAGGAGGAAG AAGTGTATGC
GGCCACGGAC GGCCATACTG TGCCTTCCCA TACACTGCCT CGTATAAAGA GTCCTCCTTC TTCACATACG

         500                                                  HaeII
GGCAGAGCGT CTGGATAAAA TCCGCCGCCA GAACCGGATA AAGGCAGAAC TTCAGGCCGT GCTTGATGAA
CCGTCTCGCA GACCTATTTT AGGCGGCGGT CTTGGCCTAT TTCCGTCTTG AAGTCCGGCA CGAACTACTT

           pWD71
CAATAAAAAA GCCTCCTTCA CACTAAAGGA GGCGGAAAAA TAAGGCTTCT CTGAAACCTT CTTCCATACC
GTTATTTTTT CGGAGGAAGT GTGATTTCCT CCGCCTTTTT ATTCCGAAGA GACTTTGGAA GAAGGTATGG

                         HaeIII                           700
CGGCAGAAGA AGGTTATAAA AATTTATAAC ACATTCACCG GCCTGATTAA AGGGGGAAGG AAAATGAACG
GCCGTCTTCT TCCAATATTT TTAAATATTG TGTAAGTGGC CGGACTAATT TCCCCCTTCC TTTTACTTGC

GAACAGGGCG TGATTC
CTTGTCCCGC ACTAAG
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Figure 3. DNA sequence of the R100 DNA containing FinO. The R100 DNA in pWD51 is numbered 1 to 712. The DNA immediately upstream of it is numbered -1 to -202. The position marked by "pWD71" is the rightmost limit of the R100 DNA in the finO⁺ BAL31 digested plasmid of that name. The line from 572 to 558 marks a potential lambda attachment site. Other lines mark regions of strong inverted repeats.

was used as a test for finO in the transformants, all colonies resistant to f1 (i.e. FinO⁺ phenotype) proved instead to be cured of the F' (see Methods). Transfer of R100-1 out of these derivatives was not inhibited indicating that the presumed finO⁺ recombinants were now FinO⁻. This finding suggests that the finO gene mutated to finO⁻. Accordingly, we could not establish any relationship between the 20,600 dalton protein and finO.

DNA sequence

All of the finO activity of R100 was contained on the R100 fragment in pWD51. The sequence of this fragment was determined, and the results are shown as bases 1 through 712 in Fig. 3. An additional 202 bases were also

PLASMID	SEQUENCES ACROSS MUTATION	NUMBER OF BASES ADDED (+) OR DELETED (-)	F'LAC TRANSFER
	JUNCTION		

A) MUTATIONS AT THE XMAI SITE (148)

	58 158		
PWD55	AAACACCGACCCGGGAAGCA	(-)81L	0.41
PWD56	123 158 AAGCAGAAACCCGGGAAGCA	(-)16L	1.6
PWD58	143 158 GGCTGCCCGGCCGGGAAGCA	(+)4L	0.05
PWD59	139 171 AGAAGGCTGCCCGGCAGCAA	(-)13R	0.004

B) MUTATIONS FROM BAL31

LEFT END

	PBR322 R100		
PWD73	49 13 TAAACTACCGTCATGACAGA	(-)3	0.0001
PWD75	60 28 TTTAACTGTGAACGACCGGT	(-)18	14.7
PWD76	76 24 CTGACTGCGTCAGAAACGAC	(-)14	1.2

RIGHT END

	R100 PBR322		
PWD70	544 91 AGGCCGTGCTGTGTATGAAA	(-)163	13.2

Figure 4. DNA sequences across mutation boundaries of several finO mutants. In (A), the vertical line marks the boundary between unchanged fragment and the region treated with DNA polymerase I. In (B), the line marks the boundary between the BAL31-digested ends of plasmid pBR322 DNA and the BAL31-digested ends of the R100 DNA. In all examples the R100 DNA was that present in pWD51. L (left) and R (right) indicate the direction of the deletion (-) or addition (+).

determined, and these are shown as negatively numbered bases. All of the experiments below that describe our attempts to define the limits of the finO gene used the 712 base fragment exclusively, and all numbering used refers to the numbers shown in Fig. 3.

Open reading frames (orfs)

Computer analysis of the sequence shown in Fig. 3 showed the presence of a large orf in the top strand that began with the ATG at base number 6 and ended with the TAA at base number 564. These codons are boxed in Fig. 3. This orf would encode a protein of 21,268 daltons, consistent with the protein seen in Fig. 2. Several smaller orfs and several sets of potential transcription initiation signals can be detected in both strands, but we have no data to indicate that any are significant.

Inverted repeats

We did find unusual the large number of inverted repeats in the total sequence. There were 31 of these, uniformly distributed over the sequence, that have a Gibb's free energy $\leq$ -11 kcal (loop size limit=300 bases) (calculated with Microgenie Program). If the loop size was reduced to 30, six inverted repeats remained, and all had values $\leq$ -13 kcals. One 11 base-long inverted repeat beginning at base 178 had a perfectly matched repeat beginning

at base 290 (-26.2 kcal).   These two sequences are marked on Fig. 3 with thin arrows.   [An imperfectly matched but still strong (-13 kcal) repeat of the sequence beginning at 179 begins at base 206].   An 8-base long perfectly matched repeat was found from bases 571 to 578 and 586 to 593.   This is marked on the bottom strand with heavy lines.   RNA from the bottom strand of both sets could form stem-loop structures that would be immediately adjacent to a long sequence of U residues, a characteristic of rho-independent termination signals.

Lambda att site

During the isolation of lambda insertions into R100 (15), it was found that lambda inserted into R100 so as to inactivate finO with a relatively high frequency.   Scanning the sequence in Fig. 3 for homologies to the lambda attachment site revealed two locations on the bottom strand.   The lambda att site sequence is GCTTTTTTATACTAA.   One sequence which has an 11/15 homology with this is marked beginning at base 558, and a second with 10/15 homology begins at base 350.

Mutations of the pWD51 fragment that affected FinO

We were not able to subclone finO with any single restriction enzyme to a single restriction fragment smaller than the HinfI fragment of pWD47 (Fig. 1). We made finO mutations by digesting pWD51 with XmaI, treating it with the Klenow fragment of DNA polymerase I, and religating it.   This treatment produced several kinds of mutations: simple "fills" of the 5' overhang, blunt-ended religations, and deletions to the right and left of the XmaI site with and without accompanying fill-in reactions on the undigested side.   These derivatives were sequenced across the site of mutation beginning in an unmodified area at least 50 bases upstream of the mutation and continuing at least 50 bases beyond where the sequence resumed the parental form.   One mutant (pWD59) was sequenced through the next in-frame translation termination signal.   The principal mutations are listed in Fig. 4 together with their critical sequences and FinO activities.   Except for pWD55, all mutations at the XmaI site introduced frame shifts that would lead to early termination of the large orf.   The orf of pWD58 ended at the TGA at 227-229 (Fig. 3), and the orf of pWD59 ended at the TGA at 208-210 (Fig. 3) (determined by direct sequencing).   The former would encode a protein of 75 amino acid residues and the latter one of 63 residues.   The first 49 residues of both were identical to the wild type (R100) orf.   Both wild type orf and these mutants had a high percentage of basic amino acids in this region, with pWD59 having the most (21

out of 63 residues Lys or Arg and only 5 Glu or Asp). Both pWD58 and pWD59 retained FinO activity, with that of pWD59 being nearly wild type level.

BAL31 digestions

The limits of finO gene activity on the cloned R100 DNA were explored by limited BAL31 treatment of ClaI digests of pWD64 and pWD65 DNA. Since the ClaI site is located in the pBR322 DNA, 25 bases from the EcoRI site, BAL31 digestion of ClaI-cleaved pWD64 DNA should produce deletions into the "right" side of the R100 DNA as shown in Fig. 1 and BAL31 digestion of ClaI-cleaved pWD65 DNA should produce deletions into the left side. pWD64 derivatives that retained the HaeIII site (GGCC) at base 545 but lost the HaeIII site at base 670, and pWD65 derivatives that retained the RsaI site (GTAC) at base 27 were mixtures of FinO$^+$ and FinO$^-$ types. Sequences across the mutated regions of representative plasmids are shown in Fig. 4. pWD73, pWD75, and pWD76 were derived from pWD65 and pWD70 was derived from pWD64. The "left" end of R100 DNA begins at base 1 in Fig. 3 and is adjacent to the beginning of the large orf. The results showed that removal of three R100 bases in front of the orf and the ribosome binding site immediately upstream caused no change in FinO activity, i.e. pWD73 had FinO activity identical to pWD47. We concluded that pWD73 contained the minimum amount of R100 DNA needed for full FinO activity. Removal of the first three codons of the orf (pWD76) caused some loss of FinO function, while deletion of an additional four bases produced a FinO$^-$ plasmid (pWD75). At the "right" or HinfI end, removal of the last 13 bases of the orf (pWD70) totally inactivated finO. The sequence of pWD70 showed that the dele- tion and religation had connected the orf to a long orf in pBR322 such that the new large orf would encode 580 amino acid residues. Accordingly, we do not know if deletion of the last few residues of the putative finO orf caused the loss of finO gene activity or whether attachment of a large useless car- boxy end caused the loss of activity.   Deletions that stopped short of entering the orf removed as much as 130 bases of the cloned fragment (see pWD71, Fig. 4) without affecting activity. The extent of this last deletion was determined solely from restriction analyses.

DISCUSSION

The simplest interpretation of the sequence presented in Fig. 3 would be that the active R100 finO gene product is the 21,268 dalton protein, but several observations make this questionable: i) The isolation of several internal deletion mutations of the cloned fragment that changed the reading

frame of the orf to end at 63 to 75 amino acid residues without destroying the
finO activity; ii) The finding that orientation of the R100 fragment of pWD51
in either direction in pBR322 or pBR325 has no effect on FinO activity; iii)
TAC-promoter derivatives that synthesized a 20,600 dalton protein were FinO⁻.
(It remains possible that the finO gene or a gene necessary for its expression
was mutated in these strains). We do not have any data that would support any
other orf as the gene product.

Another interpretation, at present equally likely, is that the finO gene
is made from the bottom strand. If we invoke transcripts from the bottom
strand as possible finO transcripts, it is possible that there are two such
transcripts, one ending near base 175 and the other in the negative region of
the sequence in Fig. 2. In this case, the differing FinO properties of the
deletions around the XmaI site might be explained by having the leftward dele-
tions remove the promoter site for the second transcript. The rightward dele-
tion in pWD59 would retain full activity because it only removed surplus DNA
between the terminator at 175 and the next promoter, somewhere left of the
XmaI site. Obviously, if this hypothesis applies, the second transcript in
the bottom strand of pWD51 might end to the left of base 1 in Fig. 3. In that
case, all sequences necessary for activity would reside on the R100 DNA in
pWD51. Since the R100 fragment in pWD51 was longer than that in the fully
FinO⁺ pWD73, the eight-fold difference in FinO activity between pWD51 and
pWD47 probably derived from vector differences. The eight-fold difference is
not likely to be associated with the loss of the ribosome binding site in the
top strand of pWD51, since the fully active pWD73 also has no ribosome binding
site. Experiments are still in progress to test these various possibilities.

Careful screening of the sequence for significant homologies with the R100
traJ and traM genes produced some homologies. The most interesting was that
11 of 12 bases from base 487 through base 498 (Fig. 3) were homologous with a
13 base stretch of R100 traJ RNA from base 537 to 549 in the sequence for R100
traJ (8). This finding provided the first sequence data that supports the
possibility that finO and finP may act on the same site. Several other homo-
logies with R100 traM were found, but the significance to the mode of action
of finO is not obvious.

In conclusion, we have shown here that the sequence of R100 DNA containing
the finO gene contained an orf that could encode a protein of 186 amino acid
residues, but we have no supporting data that such a protein is the finO pro-
duct. We do show that internal deletions that would produce shortened "finO"
proteins with only 49 amino-terminal residues identical to the wild type pro-

tein still retained FinO activity. Mutants of this kind interestingly maintain the strongly basic character of the amino end of the wild type orf. Thus, our data do not clearly define the nature of the _finO_ product and do not indicate how _finO_ controls translation of the _traJ_ transcript (3) and also increases the amount of hybridizable _finP_ gene product (Dempsey, submitted for publication).

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