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Oligonucleotide-directed mutagenesis of gene IX of bacteriophage M13

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ABSTRACT

The synthetic oligodeoxyribonucleotide pCGAAAGACTACAC has been applied as a site-specific mutagen to introduce a T → G transversion mutation at nucleotide position 1223 of the M13 DNA sequence. The *in vitro*-induced conversion of a TAT codon into a TAG codon at this position resulted in gene IX mutants with an amber mutant character thereby confirming that this reading frame defines a gene of an essential phage protein. The gene IX amber mutants obtained grew well on SuI(Ser) and SuIII(Tyr) suppressing strains but could not be propagated on SuII(Gln) and SuVI(Leu) strains. Complementation studies show that amber mutants in genes V and VII exert a polar effect on gene IX expression suggesting that these three contiguous genes form an operon. In addition, we demonstrate the *in vitro* synthesis of gene IX-protein in a coupled transcription-translation system.

INTRODUCTION

The nucleotide sequence of bacteriophage M13 DNA has learned us that the region between genes VII and VIII, which previously has been considered as a leader sequence of the major coat protein mRNA (1), could theoretically comprise a gene coding for a small polypeptide of 32 amino acids only (2,3). Recent studies on the structure of filamentous coliphages have indicated that this polypeptide, named gene IX-protein, really exists and forms a minor constituent of the phage particle (4-6). Simons *et al.*(4,5) and Grant *et al.*(7) further demonstrated that this protein and gene VII-protein are located at one end of the phage filament where they are present in 3-4 copies only. They also showed that at the opposite end about 5 copies are present of two other phage-encoded proteins, namely the products of genes VI and III.

Their location at the ends of the filament suggests that these minor capsid proteins exert a crucial role during the process of phage assembly at the host cell membrane. Detailed studies on the function of gene IX in phage morphogenesis, however, are greatly hampered by the fact that no con-

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ditionally-lethal mutants are known which map in this particularly small gene IX region. We therefore decided to construct such mutants by site-directed mutagenesis (8-13) using a synthetic oligodeoxyribonucleotide which mismatches at a single nucleotide to a specific complementary site on wild-type M13 viral DNA. After *in vitro* incorporation into closed curcular duplex DNA by elongation with DNA polymerase I and ligation followed by transfection of competent *E.coli* cells, amber mutants were selected which on the basis of their nucleotide sequence have their mutation in the established reading frame of gene IX.

We report here the construction and characterization of an *in vitro* induced amber gene IX mutant and its properties in genetic complementation tests. Our data show that the proximal genes V and VII exert a polar effect on gene IX expression, and consequently, synthesis of gene IX-protein is dependent on faithfull expression of genes V and VII. In addition, this mutant enabled us to demonstrate the synthesis of gene IX protein *in vitro*.

MATERIALS AND METHODS

Bacteria and phages

The *E.coli* strains KA798(*sup*$^+$), KA805(*sup*D), KA806(*sup*E), KA807(*sup*F) and KA809(*sup*P) used in this study were constructed by introduction of the F-plasmid in XA-strains, which originate from J.H.Miller (14). The M13 amber mutant phages *am*5-H1, *am*7-H2 and *am*8-H1 were originally obtained from Dr. D. Pratt. Phage M13 wild-type was from our own stock.

Enzymes

The large fragment of *E.coli* DNA polymerase I was obtained from Boehringer, Mannheim. T4 DNA ligase, a kind gift of P.van Wezenbeek, was prepared from an induced lysogen of λT4*lig* phage NM989 (15). T4-polynucleotide kinase was purchased from P-L Biochemicals and the restriction endonucleases used in this study were from New England Biolabs.

Isolation of DNA

Viral DNA was isolated from purified M13 phages by the hot phenol/chloroform-SDS extraction method described by Marvin and Schaller (16). M13 RF-I DNA was isolated from infected cells according to the method of Clewell (17) for isolation of plasmid DNA. M13 DNA restriction fragments were fractionated on 5% polyacrylamide gels containing E-buffer (40 mM Tris-HCl, 20 mM sodium acetate, 2 mM EDTA, pH 7.8) and eluted electrophoretically from gel segments in 50 mM Tris-borate, 1 mM EDTA, pH 8.3 with the aid of an ISCO sample concentrator. The DNA in the eluate was recovered by ethanol precipi-

tation.

Oligonucleotide-primed synthesis of M13 RF-IV DNA

Primed synthesis of covalently-closed heteroduplex DNA was performed essentially as described by Gillam and Smith (11) with minor modifications. Wild-type M13 viral DNA (0.25 pmol) and 5'-[^{32}P]-labelled oligodeoxyribonucleotide primer (10 pmol) in 2 µl of 40 mM Tris-HCl, 100 mM NaCl, 20 mM $MgCl_2$, 2 mM mercaptoethanol, pH 7.5 were incubated for 10 min at 45°C and annealed for 20 h at 4°C. Then 1 µl of dNTP-mix, consisting of 66 mM Tris-HCl, pH 7.5, 33 mM $MgCl_2$, 3 mM mercaptoethanol, 2.5 mM of each dATP, dCTP, dGTP, dTTP and 1.2 mM rATP was added, followed by 0.5 units of *E.coli* DNA polymerase I (Klenow) and 2.5 units of T4 DNA ligase. The total reaction volume was 5 µl. After incubation for 20 h at 4°C, the reaction mixture was diluted with 10 µl of TE-buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5) and incubated with 10 µg of ethidium-bromide for 1 h at room temperature in the dark (18). Covalently-closed duplexes (M13 RF-IV DNA) were isolated after electrophoretic fractionation of the reaction mixture on 1% agarose gels in E-buffer containing 2 µg/ml of ethidium-bromide and subsequent autoradiography.

Transfection and plaque assay

Gel segments containing ^{32}P-labelled RF-IV DNA were excised and the DNA was eluted electrophoretically as described. The eluate was extracted twice with phenol/chloroform and the DNA was concentrated by ethanol precipitation. The preparation of competent *E.coli* KA807(*sup*F) cells and the subsequent transformation procedure was essentially as described by Kushner (19). The transfected cells were plated for infective centers on *E.coli* KA807 cells as the indicator strain. Plaques were tested by plating 10 µl of each plaque suspension (obtained by needle-picking the plaque and tranfer into 200 µl of TE-buffer) on a KA798(*sup*$^+$) host and on a KA 807(*sup*F) host.

Marker rescue experiments were performed by the method of Taketo (24) as described by van den Hondel *et al.*(25).

DNA sequence analysis

Labelling of 5'-terminal ends of oligodeoxyribonucleotide primer and restriction fragments was carried out as described previously (20). Sequence analysis of the synthetic oligodeoxyribonucleotide was carried out by partial digestion of the DNA with snake venom phosphodiesterase as described by Maniatis *et al.*(21). The partial digestion products were characterized by two-dimensional fingerprinting using the homo-mix V of Jay *et al.* (22) for the second direction. Sequence analysis of M13 restriction fragments was performed by the method of Maxam and Gilbert (23).

Cell-free protein synthesis

Cell-free protein synthesis, programmed with either M13 wild-type RF or amber mutant RF, was carried out as described by Konings (26). The synthesized polypeptides were analysed on vertical 15% polyacrylamide-SDS gels containing 8 M urea as described previously (4).

RESULTS

The general outline of our approach to construct M13 gene IX amber mutants is shown in Fig. 1. The oligonucleotide used to induce the amber mutation in wild-type M13 viral DNA is the triscaidecadeoxyribonucleotide pCGAAAGACTACAC. It was chosen as its complementary sequence occurs only once in the M13 viral DNA sequence, namely at nucleotides 1218 to 1230 of the gene IX sequence. The underlined nucleotide, eighth from the 5' end, mismatches with a T at position 1223 of the viral DNA sequence. The 13-mer was synthesized by the chemical phosphotriester method (27) and its nucleotide sequence was confirmed by two-dimensional fingerprinting (Fig. 2) and direct DNA sequencing methods. The experiments gave the anticipated sequence and showed that the 13-mer was virtually free of contaminants.

Since quite short oligodeoxyribonucleotides with one mismatched base pair still form stable duplex structures, the synthesized 13-mer was used as a primer for elongation with *E.coli* DNA polymerase I (Klenow-fragment) in the presence of T4 DNA ligase. For this purpose we used primer molecules labelled with ^{32}P at their 5' ends. Circularly closed relaxed DNA molecules

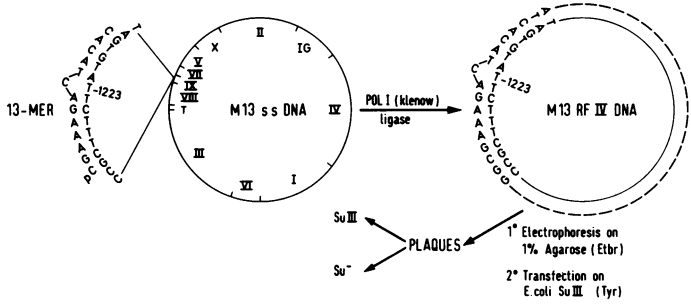

Figure 1 *In vitro* selection of M13 gene IX amber mutants using oligonucleotide pCGAAAGACTACAC. The "mutant" oligonucleotide which mismatches at nucleotide 1223 of gene IX was used as primer for *E.coli* DNA polymerase I (Klenow) in the presence of T4 DNA ligase. Closed circular heteroduplex DNA was electrophoretically isolated and then used to transfect competent *E.coli* (SuIII)-cells. After plating, amber mutants were selected by a parallel plaque assay on a Su^- and a $SuIII^+$ host.

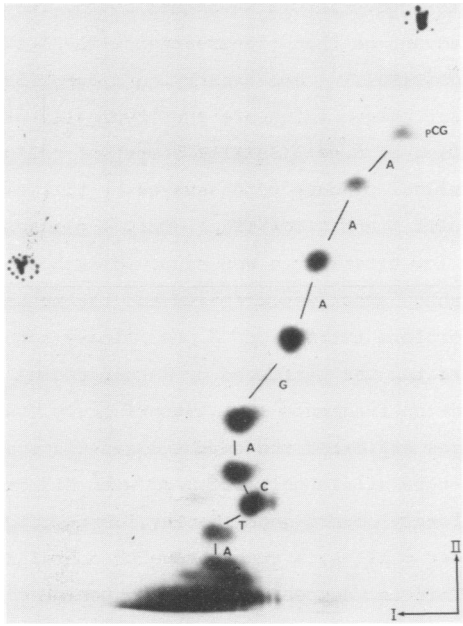

Figure 2 Two-dimensional sequence determination of the 5'- [^{32}P]-labelled synthetic oligodeoxy- ribonucleotide pCGAAAGACTACAC. The first dimension was electrophoresis on cellulose acetate strips (pH 3.5). The second dimension was homochromatography on DEAE thin-layer plates using homomix V (22). The positions of the Xylene xyanol (top) and Orange G marker (left) are indicated.

(RF-IV DNA) were isolated by fractionation of the reaction mixture on 1% agarose gels containing 2 µg/ml of EtBr (18). Under these electrophoretic conditions, RF-IV DNA molecules migrate slightly faster than supercoiled RF-I DNA and are well separated from non-ligated RF molecules containing a gap or nick in their complementary strand and from the excess of single-stranded viral DNA template (data not shown). Using this method, enrichment and selection of RF-IV molecules by S1-nuclease digestion (11), filtration on nitrocellulose filters at high salt concentrations (8) or banding in CsCl density gradients (12), applied by others, can be circumvented.

Regions of the gel containing ^{32}P-labelled RF-IV DNA were excised, the DNA was electrophoretically eluted from the gel segments and further purified as described under Methods.

Isolation and characterization of amber mutants

Site-specific mutagenesis with the synthesized 13-mer is expected to induce a T → G conversion at nucleotide 1223 in M13 viral DNA (Fig. 1). This

changes a TAT tyrosine codon into a TAG amber codon. The induction of such a mutation has the advantage that transfection with RF-IV DNA can now be carried out with *E.coli*(SuIII$^+$) cells carrying a tyrosine suppressor, resulting in progeny mutant phages which are phenotypically of wild-type character

After transfection of *E.coli*(SuIII$^+$) competent cells with ^{32}P-labelled RF-IV DNA, 160 individual plaques were assayed by titrating each plaque suspension on *E.coli*(SuIII$^+$) and *E.coli*(Su$^-$). Only 4 plaques gave high titers on the *Su*$^+$ host and low titers on a non-suppressing host. Of these four mutants a purified phage stock was prepared and the mutant viral DNA was isolated by phenol/chloroform extraction. A preliminary screening for the gene IX amber mutant character was performed by marker rescue experiments with purified M13 restriction fragments (25). Surprisingly, only the viral DNA of three mutant phages exhibited the expected genetic specificity in that salvage of the wild-type allele only occurred with M13 fragment *Hin*fI-A, a fragment which completely covers gene IX. The fourth phage mutant could only be rescued by fragment *Hap*II-A, a fragment which contains the genetic information of gene IV. This is unexpected as a computer search shows that the *Hap*II-A sequence does not comprise a region which is complementary to the primer sequence (see Discussion).

In order to show conclusively that the method applied here induces the intended mutation in gene IX, we have determined the nucleotide sequence change in the induced amber mutants obtained. Figure 3 shows the relevant portion of a sequencing gel of one of the three amber mutant DNAs which were rescued by the M13 *Hin*fI-A fragment. The sequence presented starts with the ATG initiation codon of gene IX. The only difference between this sequence run and that of wild-type M13 DNA is noted at nucleotide 1223. In the amber mutant, called *am*N-113 (left panel), a T → G conversion has taken place which has changed the TAT tyrosine codon into a TAG amber codon. The other two amber mutants, called *am*N-18 and *am*N-22, showed an identical sequencing ladder as *am*N-113 in that they also revealed a T → G conversion at nucleotide 1223 (data not shown).

Our data demonstrate that the oligonucleotide pCGAAAGACTACAC has induced the intended mutation in that the sixth codon of gene IX has been converted into an amber codon. The isolation of amber mutants in this gene further demonstrate that gene IX forms an essential gene of phage M13 the protein product of which is needed for progeny phage production.

We did not yet characterize the base change involved in the amber mutant ,called *am*N-129, which was rescued by the *Hap*II-A fragment.

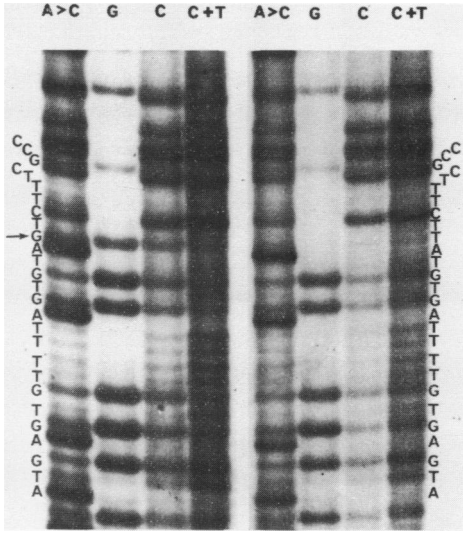

Figure 3 Autoradiograph of DNA sequencing gels obtained after partial chemical degradation of the right hand 5'-terminal end of fragment *Hha*I-H of *am*N-113 RF-DNA (left panel) and of the same fragment derived from wild-type RF-DNA (right panel). The nucleotide positions 1206-1232 of the viral strand of these fragments are designated. The arrow indicates the expected T → G conversion at position 1223 of the M13 gene IX sequence.

Properties of the in vitro-induced amber mutants

To garantee the production of progeny mutant phages, the transfection with primed RF-IV DNA was not carried out on the conventional serine (SuI^+) but on the tyrosine ($SuIII^+$) suppressing strain. This precaution appeared to be unnecessary. The data presented in Table I show that the mutants *am*N-113, *am*N-18 and *am*N-22 not only gave progeny phages on strain KA807 but also on strain KA805 with equal efficiency. These mutants could not propagated, however, on a glutamine- or a leucine-suppressing strain. The deviating character of *am*N-129 is also apparent since replacement of tyrosine by a serine or glutamine residue is now tolerated but incorporation of a leucine residue at the amber locus does not restore the protein function.

By genetic complementation studies Lyons and Zinder (28) were first to demonstrate that nonsense mutants in gene V exert a polar effect on gene VII expression. As gene IX is distal to gene VII we found it of particular interest to investigate whether this polarity also includes gene IX. For this reason standard complementation tests were carried out. Our results, which are summarized in Table II, demonstrate that the mutual complementation of

TABLE 1

Suppression of the *in vitro*-induced amber lesions in various suppressing hosts

Strain	SuI(Ser)	SuII(Gln)	SuIII(Tyr)	SuVI(Leu)
Mutant				
*am*N-18	2.8×10^{12}	3.3×10^{7}	2.6×10^{12}	1.2×10^{7}
*am*N-22	2.5×10^{12}	1.6×10^{7}	2.5×10^{12}	1.3×10^{7}
*am*N-113	3.2×10^{12}	2.4×10^{7}	2.8×10^{12}	1.6×10^{7}
*am*N-129	7.9×10^{12}	6.3×10^{12}	8.0×10^{12}	3.8×10^{7}

Suppression is expressed as the efficiency of plating on the various strains

defined amber mutants in gene V, VII and IX is very low but that each of these mutants complement normally with a gene VIII amber mutant. These data strongly suggest that the contiguous genes V, VII and IX form anoperon with gene V amber mutants polar on genes VII and IX and with gene VII amber mutants polar on gene IX.

In vitro synthesis of gene IX-protein

With the aid of a DNA-dependent cell-free protein synthesizing system up to now only the products of six out of the eight known M13 genes have been identified (29,30). For unknown reasons and despite extensive efforts the products of genes VI and VII have not been found. On the other hand, M13 DNA directs the *in vitro* synthesis of two polypeptides (mol.wt. 12,000 and 3,500) which could not be assigned to known M13 genes (29-31). One originates from the hypothetical gene X, the nucleotide sequence of which forms the C-terminal part of gene II. The origin of the small polypeptide remained

TABLE 2

Genetic complementation values between various amber mutants in genes V, VII, IX and VIII

	*am*5-H3	*am*7-H2	*am*N-113	*am*8-H1
*am*5-H3	0.001	0.002	0.01	0.25
*am*7-H2	0.002	0.001	0.003	0.07
*am*N-113	0.01	0.003	0.001	0.23
*am*8-H1	0.25	0.07	0.23	0.001

The values given are the ratios of plaque formers to total cells plated, corrected for wt-revertants, found in single infections of both phages. Complementation is classified as strong when the ratio is greater than 0.15, weak when the ratio is between 0.01 and 0.15 and negative when the ratio is less than 0.01.

obscure. As the nucleotide sequence of gene IX predicts that its translation product is only 32 amino acids long (3,20) and gene IX amber mutants are now available, we have carried out coupled transcription-translation analysis to find out whether the 3,500 dalton protein could be the product of this gene. As shown in Fig. 4, protein synthesis under the direction of RF-DNA derived from *am*N-113 gives rise to a number of M13-specified products which are also present among the products synthesized under the direction of wild-type M13 RF-DNA. Since [^{35}S]-cysteine was used as label, synthesis of gene VIII-protein can not be detected as this amino acid is absent in this protein. The sole difference is in the lower part of the gel where the absence of the 3,500 dalton protein is noted when synthesis is programmed by the mutant RF. Our conclusion therefore is that the *in vitro* synthesized 3,500 dalton protein is the product of gene IX.

As judged from the protein synthesis data (Fig. 4), the mutant *am*N-129 bears its mutation in gene IV. Protein synthesis under the direction of *am*N-129 RF-DNA shows the disappearance of the wild-type gene IV-protein (48,800 daltons) and the appearance of an abortively terminated polypeptide of about 40,000 daltons. The *in vitro*-induced amber mutation is therefore located in the C-terminal part of gene IV. It is worth mentioning that this

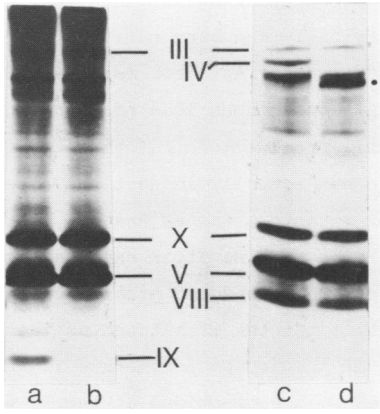

Figure 4. SDS-8M urea polyacrylamide gel electrophoresis of the M13-specified proteins synthesized under the direction of : (a) and (c), wild-type M13 RF-DNA (b), *am*N-113 RF-DNA (d), *am*N-129 RF-DNA Synthesis reactions (a) and (b) were carried out in the presence of [^{35}S]-cysteine, reactions (c) and (d) in the presence of [^{14}C]- proline. Roman numerals indicate the products specified by the M13 genes. The position of the prematurely terminated *am*-IV protein is indicated with a black dot. Note the absence of gene VIII-protein in the left lanes and of gene IX-protein in the right lanes.

experiment substantiates our conclusion on gene IX-protein. Since the gene IX sequence lacks proline, synthesis of this protein can not be detected.

DISCUSSION

Nearly all phage M13 amber mutants used at present have been selected after mutagenesis of mature M13 phage particles with hydroxylamine (23). This cytosine-specific mutagen is *a priori* unsuitable for the induction of amber mutants in gene IX as the nucleotide sequence of this gene lacks any CAG codons which can be converted into a TAG amber codon. On the other hand, the gene IX sequence comprises two TAT (Tyr) codons. They are attractive targets for the induction of amber mutations since selection of such mutants can be performed with tyrosine-suppressing strains warranting the production of mutant phage progeny. By *in vitro* incorporation into closed circular duplex DNA of an oligodeoxyribonucleotide complementary to a defined gene IX region except for one mismatch at the third base of its first TAT codon followed by transfection of *E.coli*(SuIII$^+$) cells, we have been able to induce and select such gene IX mutants.

With respect to mutant N-129, the amber locus of which is in the distal quarter of gene IV, we do not know yet which nucleotide change has taken place. The region concerned does not contain a sequence which is complementary to the 13-mer. If G-T base pairing is taken into account, there are several regions which theoretically can base pair with 8 out of 13 nucleotides but in all cases the induction of an amber mutant lesion is accompanied by base changes in the contiguous codons leading to amino acid replacements. DNA sequence analysis will provide more information on which part of the oligodeoxyribonucleotide has actually induced the amber mutation.

The number of amber mutants obtained was low as compared to the results of other groups (8,9,11). We have no clear explanation for this but the reduced efficiency could be due to reduced priming efficiency, if this was limiting, or due to editing out the mismatched nucleotide by the intrinsic 3' exonuclease activity of *E.coli* DNA polymerase I. The mismatched nucleotide in the 13-mer is followed by 5 nucleotides at the 3' end. Since Gillam and Smith (11) demonstrated that efficient specific mutagenesis already can be achieved with primers containing as few as seven nucleotides provided the mismatch is followed by one or preferably two nucleotides at the 3' end, we expect that in our case editing out of the mismatch by the 3' exonuclease has not contributed significantly to the reduced efficiency. On the other hand, a crucial step in our procedure is the immediate ligation of relaxed

duplexes without concomitant repair of the mismatch by the 5' exonuclease activity of *E.coli* DNA polymerase I. For this purpose we used the large (Klenow) fragment of this enzyme. Since control experiments showed that closed duplexes were formed even when the primer was deprived of its 5'-phosphate, we cannot exclude that the reduced percentage of amber mutants scored is due to remnants of 5' exonuclease in the Klenow enzyme.

By Edman degradation studies we recently provided evidence that one of the minor capsid proteins of the M13 phage particle has an N-terminal amino acid sequence which fits the DNA sequence of gene IX (5). The production of an amber codon, starting at nucleotide 1221 and which is lethal in Su^- hosts, constitutes a formal synthetic confirmation that this reading frame does define the gene of an essential protein. Furthermore, we now have been able to demonstrate and identify this essential protein by *in vitro* DNA-dependent protein synthesis under the direction of M13 RF-DNA.

The most interesting conclusion of our study is that synthesis of gene IX-protein is under strict control of the expression of its proximal genes V and VII. This is not only concluded from the genetic complementation data presented here but finds now strong support also from our *in vivo* studies with *E.coli* mini-cells (31). In this system the synthesis of a 3,500 dalton protein, the nature of which was ambiguous, became apparent in case such minicells harboured wild-type M13 RF. It was absent, however, in minicells harbouring RF derived from gene V and gene VII amber mutants. Now this protein has been identified as the product of gene IX, it augments our conclusion that this cluster of genes forms a regulatory unit. The mechanism which regulates the expression of these genes will be described in detail elsewhere (G.Simons *et al.*, in preparation).

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