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Genetics

Methylation induced premeiotically in *Ascobolus*: Coextension with DNA repeat lengths and effect on transcript elongation

(*Ascobolus immersus*/cytosine methylation/filamentous fungus/gene silencing)

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ABSTRACT **The effect of duplications of gene fragments on the triggering of DNA methylation induced premeiotically (MIP) was studied in the sexual progeny of strains harboring, in addition to the resident *met2* gene, a fragment of this gene inserted at an ectopic position. Cytosine methylation of the resident gene was checked for each of the eight duplications tested. Methylation was always found and it was coextensive with the length of the duplications. Silencing of the resident gene was triggered by duplications of segments corresponding to the region 5′ to the open reading frame, to only the open reading frame, or to segments beginning 0.87–1.2 kb downstream from the transcription start. Silencing was accompanied by either the absence of transcripts or the presence of truncated transcripts, which suggests that methylation acts on transcript elongation.**

In the filamentous fungus *Ascobolus immersus*, repeated genes artificially obtained by integrative transformation frequently lose their expression during the sexual phase (1–3). This inactivation is not due to mutations, and affected genes are merely silenced. Indeed, no mutation in inactivated genes is found by DNA sequencing and all of the inactivated progeny are able to return, under selective pressure, to a phenotype which is identical to that of the parental transformant (4). Silencing is associated with methylation of most or all cytosine residues within the repeats (2–4). This process, induced in the haploid nuclei during the period between fertilization and karyogamy (3), has been named MIP, which stands for "methylation induced premeiotically" (4). We use this acronym to designate gene silencing as well as DNA methylation. The absence of mutation in silenced "MIPed" genes distinguishes the MIP process from RIP (repeat-induced point mutation), an inactivation process occurring in *Neurospora crassa* which is also triggered premeiotically by DNA repeats (5, 6). RIP usually leads to numerous mutations (7), although sometimes their number appears to be quite small (8).

When duplicated genes are at ectopic chromosomal locations, either both of the ectopic copies are affected or neither of them is. This strongly suggests that MIP triggering requires a direct recognition between the duplicated sequences (3). The finding that ectopic sequences about 5–6 kb in size are able to recognize each other with such a high efficiency raises new questions about genomic interactions between homologous sequences. Before investigating parameters that are critical for MIP (e.g., the minimal length of duplication), it was important to know whether the duplication of fragments of a gene would be sufficient for triggering gene inactivation (or methylation) and whether different parts of a gene could be used with success.

To answer these questions, we have duplicated definite subfragments of the *met2* gene and analyzed the effects of these duplications upon *met2* expression and methylation. Duplicating fragments of *met2* allowed us to investigate the effect of methylation on gene transcription.

MATERIALS AND METHODS

Strains, Media, and Genetic Procedures. *A. immersus* strains belong to stock 28 (9). The wild-type Met^+ Amd^- strain RL95 is devoid of any acetamidase gene and its mating type is *mt*−. The Met^+ Amd^- strains used as testers in crosses with strains Dup1–Dup8 were FA21 and FA59 (*b2.138, rd1.2, mt*+) and the Met^- Amd^- strain used as tester in crosses with Dup5 was FA46 (*met2.1, b2.138, rd1.2, mt*+). *b2* and *rd1* are ascospore color and ascospore shape markers, respectively. Their use was explained in ref. 3. For most media, see ref. 10. Standard *A. immersus* genetic techniques were used (11). The Amd phenotype was checked on medium containing acetamide as nitrogen source (3) and supplemented with methionine (20 μg/ml). The Met phenotype was checked on minimal medium.

Isolation and Manipulation of DNA. Small-scale DNA extractions were performed as described (2). Mycelia used for DNA extraction of Met^- Dup derivatives were grown on minimal medium plus methionine (20 μg/ml). For Southern hybridization, DNA (3 μg) was digested with at least 5-fold excess of enzymes to ensure complete digestion, digests were fractionated in 1.2% agarose gels, and gel blots were hybridized to ^{32}P-labeled probes. A 1-kb "ladder" from Bethesda Research Laboratories was used as size markers.

Isolation and Manipulation of RNA. Mycelia were grown on minimal medium plus methionine (20 μg/ml). Methionine does not inhibit *met2* transcription in *A. immersus* (12). Three-day cultures in liquid medium were used to inoculate plates overlaid with cellophane pads. Mycelia were harvested after 24 hr of incubation, washed, pressed dry, frozen in liquid nitrogen, powdered in a Waring Blendor, rapidly suspended in 7 ml of lysis buffer (0.1 M NaOAc, pH 5.2/5 mM $MgCl_2$/1% SDS/1% diethyl pyrocarbonate), and treated as described (13), except that phenol extractions were at 65°C. For Northern blots, total RNA (50 μg per lane) was electrophoresed in a 1% agarose gel containing 6% formaldehyde. Transfer of RNA to cellulose membrane (Amersham Hybond-C extra) and membrane hybridization were performed by standard procedures (14). After hybridization, membranes were washed at 50°C in 0.2 × standard saline citrate/0.1% SDS. For reverse transcriptase (RT)–PCR, 2-μg samples of the same preparations of total RNA were used in each experiment. RNA was first treated with RNase-free DNase I

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Abbreviations: MIP, methylation induced premeiotically; ORF, open reading frame; RIP, repeat-induced point mutation; RT, reverse transcriptase.

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(Pharmacia) and then reverse transcribed (15) with Superscript RNase H⁻ reverse transcriptase (GIBCO/BRL) using random hexamer primers (Pharmacia). One-tenth of the cDNA obtained was used for each PCR. Amplifications were performed in 50-μl reaction volumes containing 20 pmol of each primer, 200 μM each dNTP, 2 units of *Taq* polymerase (Bioprobe, Tustin, CA), and the buffer supplied by the manufacturer. Reactions were carried out in a Perkin–Elmer/Cetus DNA thermal cycler as follows: 45 s at 94°C, 30 s at 57°C, 90 s at 72°C for 32 cycles, with an additional 10 min at 72°C at the end of the last cycle. Fifteen microliters of the final mixture was used for gel electrophoresis. Primers corresponded to the following oligonucleotides, numbered in the 5′ → 3′ direction, according to the nucleotide numbering by Goyon *et al.* (12): A, 210–229; B, 377–395; C, 964–946; D, 1250–1268; E, 1406–1388; F, 1764–1746. Primer A is located upstream from the transcription start.

RESULTS

Duplications of Distinct *met2* Fragments Result in *met2* Silencing. We constructed strains Dup1–Dup8 (which carry the duplications 1–8, respectively) by transformation of the Met⁺ Amd⁻ strain RL95 with plasmids containing the corresponding fragment (Fig. 1). Transformants were selected for their ability to grow on medium with acetamide as sole nitrogen source. Dup strains carried the wild-type resident *met2* gene and the transgenic *met2* fragment integrated at an ectopic chromosomal site, together with the *amdS* selectable marker. The presence of the transgenic *met2* fragment and its ectopic location were checked by Southern hybridization (data not shown). To examine the effect of duplications upon the expression of the resident *met2* gene in strains that had undergone sexual reproduction, Dup strains (Met⁺ Amd⁺) were crossed with Met⁺ Amd⁻ tester strains. Asci from each cross were dissected, and spores were germinated on rich medium and then tested for their Amd and Met phenotypes. Since transformed protoplasts are generally multinucleate, transformants are likely to contain a mixture of both transformed and untransformed nuclei. Consequently, only 4 Amd⁺:4 Amd⁻ asci which had inherited the transformant

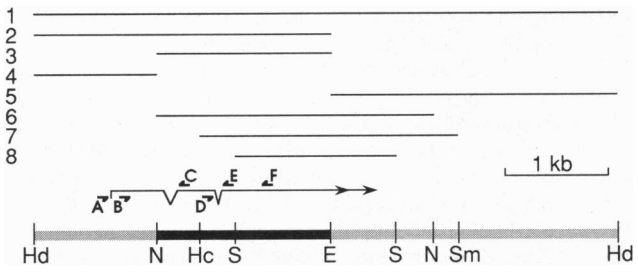

FIG. 1. Partial restriction map of the *met2* region, *met2* subfragments used in construction of Dup strains, and primers used in RT–PCR amplification experiments. E, *Eco*RV; Hc, *Hin*cII; Hd, *Hin*dIII; N, *Nsi* I; Sm, *Sma* I; S, *Sph* I. The black box corresponds to the *met2* open reading frame (ORF). The arrowed line shows the transcribed region. The two arrowheads indicate the two termination sites (12). The two V-shaped lines show the two introns. Intron 1 (from the 5′ end) is 165 nt long (12), and intron 2 is 51 nt long (L. Rhounim, personal communication), starting at nt 1319, according to the nucleotide numbering of Goyon *et al.* (12). The single-sided arrows A–F show the positions and orientations of the six primers used in RT–PCR experiments. Lines 1–8 indicate the extension of distinct segments of the *met2 Hin*dIII fragment [the sizes of which are 5.7 kb (segment 1), 2.9 kb (2), 1.7 kb (3), 1.2 kb (4), 2.8 kb (5), 2.7 kb (6), 2.5 kb (7), and 1.6 kb (8)]. These fragments were inserted in either orientation into the polylinker of plasmid pGB1. Plasmid pGB1 resulted from the insertion into vector pUC19 of the *Aspergillus nidulans Sal* I–*Eco*RI fragment (16) which carries the *amdS* gene, encoding acetamidase.

information were considered. The presence in such asci of Met⁻ derivatives showed that the resident *met2* gene had been inactivated. All Dup strains except Dup5 gave a substantial number of 4 Met⁺:4 Met⁻ asci (Table 1). As expected from previous experiments (4), all the 311 tested Met⁻ derivatives (Table 1) could revert to prototrophy, usually between one and several weeks after their transfer to minimal medium. The exceptions were Dup4 Met⁻ derivatives, which always gave reversions as early as 2 days after transfer.

No 4 Met⁺:4 Met⁻ asci were found among the 49 4 Amd⁺:4 Amd⁻ asci analyzed in the progeny of Dup5 strains (Table 1). An identical number of 4 Amd⁺:4 Amd⁻ asci were analyzed in the progeny of Dup5 strains crossed with the Met⁻ Amd⁻ tester strain. All the asci showed a 4 Met⁺:4 Met⁻ segregation, confirming the absence of Met⁻ derivatives. The failure of duplication 5 to trigger the appearance of Met⁻ derivatives could result either from a failure to cause silencing or from early reactivation. To distinguish between these two hypotheses, we tested the Met phenotype of Dup5 progeny from the cross Dup5 × Met⁺ Amd⁻ at an early stage, as soon as the spores germinated. Comparing the percentages of nongerminated spores obtained on medium with or without methionine allowed us to assess the percentages of Met⁻ spores. In this test, Dup5 did not give rise to a detectable number of Met⁻ spores. This result shows that the resident copy had not been transiently silenced.

Thus, duplicating the region 5′ to the ORF (Dup4), the ORF alone (Dup3), or fragments beginning 0.87 or 1.2 kb downstream from the transcription start (Dup7 and Dup8) was sufficient to trigger *met2* silencing. In contrast, duplicating the region 3′ to the ORF (Dup5) had no detectable effect on *met2* expression.

Methylation Is Restricted to the Duplicated Region. We first analyzed the DNA of Met⁻ Amd⁻ progenies of strains exhibiting silencing (i.e., Dup1–Dup4 and Dup6–Dup8). These strains contained only the resident silenced copy of *met2* and thus had not received the transgenic fragment through meiotic segregation (Fig. 2). *Sau*3A1 and *Mbo* I digests were analyzed by Southern hybridization. *Sau*3A1 and *Mbo* I recognize the same sequence, GATC; *Sau*3A1 will not cut the sequence if the cytosine is methylated, whereas *Mbo* I is insensitive to cytosine methylation (17). A map of the hybridizing *Mbo* I fragments from the recipient strain is shown in Fig. 3. We analyzed nine Met⁻ derivatives from one Dup1 strain. With Dup2 (and also with Dup3, -4, -6, -7, and -8), we analyzed four Met⁻ derivatives from two independent Dup strains (two from each). Met⁻ derivatives issued from strains carrying the same duplication gave the same results. Examples are given in Fig. 4.

All GATC sites were still intact in every Met⁻ derivative, since they showed the same *Mbo* I fragments as those of the recipient strain.

Table 1. *met2* silencing in Dup strain progenies

Duplication*	Met⁺:Met⁻ segregation† 4:4	Met⁺:Met⁻ segregation† 8:0	No. of Met⁻ derivatives tested
1 (1)	17	7	25
2 (4)	66	51	124
3 (4)	22	37	40
4 (3)	32	66	62
5 (4)	0	49	—
6 (2)	10	18	20
7 (2)	9	19	17
8 (2)	13	30	23

*Duplication numbers are those given in Fig. 1. The number of different Dup strains crossed is given in parentheses.
†Only 4 Amd⁺:4 Amd⁻ asci were taken into account.

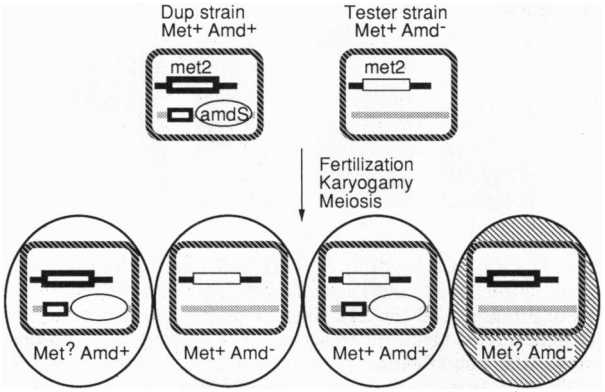

FIG. 2. Selection of the Met⁻ Amd⁻ segregants from Dup1–Dup4 and Dup6–Dup8 strains. Each Dup strain contains the wild-type resident *met2* gene and one transgenic fragment. It is crossed with the Met⁺ Amd⁻ tester strain. The *amdS* gene, present as a single copy, never becomes silenced. The four possible meiotic products (two parental and two recombinant) are shown in oval frames. Silencing is stable even once the transgene has segregated away. Thus, segregants which have inherited the resident *met2* gene from the Dup parent can be either Met⁺, in the absence of silencing, or Met⁻, if silencing occurred. Their phenotypes are indicated as "?." Only the spore represented in the shadowed frame can be Met⁻ Amd⁻, thus containing the silenced *met2* gene only, without the transgenic fragment.

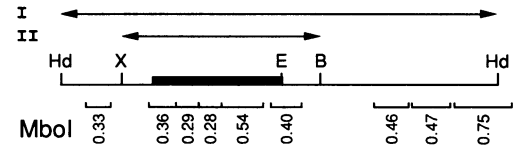

FIG. 3. Partial *Mbo* I restriction map of the *met2* region. B, *Bgl* II; E, *Eco*RV; Hd, *Hin*dIII; X, *Xba* I. Only *Mbo* I fragments detected in the experimental conditions used are shown. Positions of *Mbo* I fragments were deduced from DNA sequence data [0.54-, 0.40-, 0.36-, 0.29-, and 0.28-kb fragments (12)] or restriction mapping of the *Hin*dIII fragment [0.75-, 0.46-, 0.47-, and 0.33-kb fragments (the order of which is known, but not the exact locations)]. I and II indicate the DNA probes used in hybridization experiments. The filled box represents the *met2* ORF.

In the Met⁻ derivatives from the Dup1 strain (in which the 5.7-kb *Hin*dIII fragment carrying the whole *met2* gene had been duplicated), the expected *Sau*3A1 fragments were missing and they were replaced by larger fragments ranging up to 6 kb (Fig. 4). This shows that all the GATC sites present in the duplicated sequence had been methylated, although methylation was not accurately maintained in all DNA molecules, as deduced from the presence of large fragments of different sizes. The disappearance of the *Sau*3A1 fragments together with the appearance of a 6-kb fragment suggests that methylation is coextensive with the duplication length. In other experiments, 11 other restriction endonucleases which are sensitive to cytosine methylation also failed to cut sites located within the duplicated region of Dup1 derivatives (4).

With other Met⁻ derivatives from Dup2–Dup4 and Dup6–Dup8, all the results were also consistent with methylation extending the entire length of the region of homology. First, the size of each largest fragment was consistent with failure to cut any *Sau*3A1 site located within the duplicated sequences. Second, the missing bands corresponded to *Sau*3A1 fragments bounded by at least one GATC site internal to the duplications, whereas fragments bounded by GATC sites both of which were external to the duplications were still present. Notably, the last internal *Sau*3A1 site, 172 bp upstream from the 3′ boundary of duplication 3 (12), was not cut. With Dup4 derivatives, the largest (1.6-kb) *Sau*3A1 fragment results from the failure to cut any internal GATC sites. This fragment is less abundant than the 1.2-kb fragment, suggesting that the *Sau*3A1 site located within the duplication, 57 bp upstream from the 3′ boundary of Dup4 (12) was unmethylated in a large fraction of the DNA molecules. The largest fragments were also produced in substoichiometric amounts in Dup6 and Dup8 derivatives, suggesting again that the internal ends of the duplications were incompletely methylated.

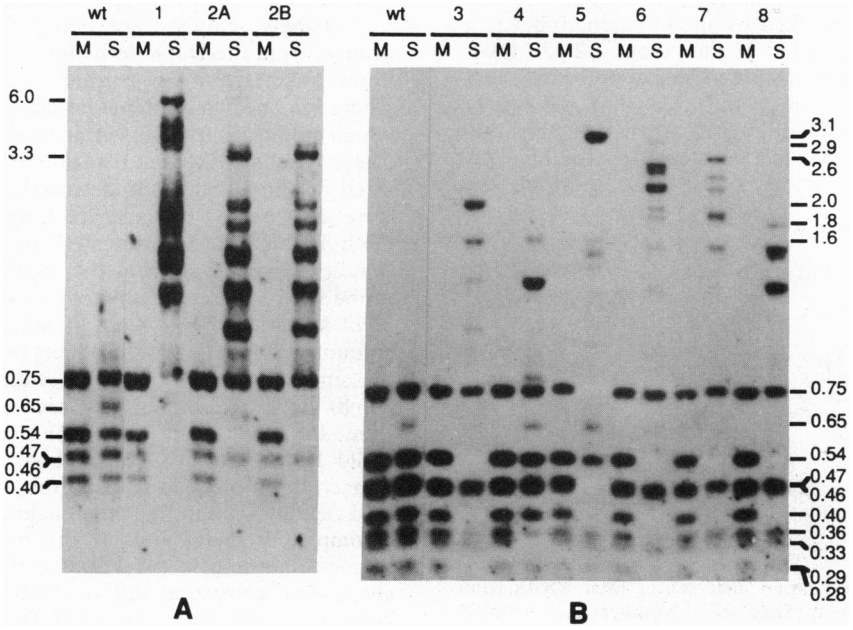

FIG. 4. Southern hybridization analysis of derivatives from Dup1–Dup8 strains. *Mbo* I (M) and *Sau*3A1 (S) DNA digests were hybridized to probe I (Fig. 3). wt, Wild-type recipient strain. Numerals 1–8 correspond to Dup1–Dup8 derivatives, respectively; 2A and 2B correspond to two Met⁻ derivatives from Dup2. Fragment sizes are in kilobases. Fragments shorter than 0.4 kb are not visible on the autoradiograph presented in *A*. Complete digestion with *Sau*3A1 was checked by reprobing with plasmid pSF5 containing the *A. nidulans* actin gene (18) (data not shown). An extra 0.65-kb fragment appears in the *Sau*3A1 control (wt) digest. It results from the partial digest of a GATC site which might contain a naturally methylated cytosine.

DNA from Met⁻ derivatives of the different Dup strains was also digested by *Hpa* II. This enzyme cleaves the CCGG site only if both cytosines are unmethylated (17). Southern hybridization confirmed results obtained with *Sau*3A1 (data not shown). CCGG sites internal to the duplications were not cut, with a few exceptions involving sites close to the boundaries. CCGG sites located outside the duplication were cut, notably one CCGG site located outside duplication 3, 16 bp upstream from its 5′ boundary (12).

All these results are consistent with methylation extending over the length of homology, with lower methylation close to the internal borders of the duplication and no detectable extension outside of it.

Methylation Without Silencing. Dup5 strains (in which the region 3′ to the ORF was duplicated) did not produce any Met⁻ progeny. DNA analysis was performed on eight Met⁺ Amd⁻ segregants from crosses of two distinct Dup5 strains with a Met⁻ Amd⁻ tester strain. All the segregants showed the same *Mbo* I fragments as those of the recipient strain. With *Sau*3A1, five out of six segregants (first cross) and one out of two (second cross) showed methylation. One example is given in Fig. 4. The size of the largest methylated *Sau*3A1 fragment (3.1 kb) indicates that here again methylation extended only the length of the duplicated region (2.8 kb) and not over the whole gene. This is confirmed by the specific disappearance of the fragments which are located within the duplicated region.

Transcript Analyses with Strains Having a Partly Methylated *met2* Gene. To investigate the effect of MIP on gene expression, we examined the transcripts of the partly methylated gene copies obtained with duplications 1, 4, 5, and 8. Northern hybridizations were performed on total RNAs (Fig. 5) with probe II (Fig. 3). No significant change appeared in the transcript pattern of the Dup5 progeny in which the region 3′ to the ORF was methylated and which had a Met⁺ phenotype. *met2* transcripts were not detected when either the entire *met2* region (Dup1) or the region 5′ to the ORF (Dup4) was methylated. Methylation of the 3′ portion of the ORF (Dup8) led to a low level of small transcripts (1.2–1.5 kb). The size of such transcripts was that expected if transcript elongation were blocked by methylation. Indeed, in Dup8 derivatives, methylation begins about 1.2 kb downstream from the transcription start. In this hypothesis, shortened transcripts might also be produced in Dup7 derivatives, where methylation begins about 0.8 kb downstream from the transcription start. We checked the hypothesis by RT–PCR amplification of distinct portions of the *met2* transcripts, from

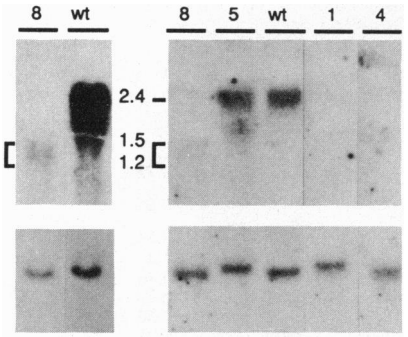

FIG. 5. Northern hybridization analyses of total RNAs from strains having differently methylated *met2*. Numbers 1, 4, 5, and 8 indicate strains derived from Dup1, Dup4, Dup5, and Dup8, respectively. The recipient strain (wild type, wt) was used as the unmethylated control. RNAs were hybridized either with probe II (*Upper*) or with plasmid pSF5 (*Lower*) containing the *A. nidulans* actin gene (18), which was used as an internal control of RNA amount. Sizes are in kilobases. The two panels correspond to separate Northern hybridization experiments using the same RNA preparations.

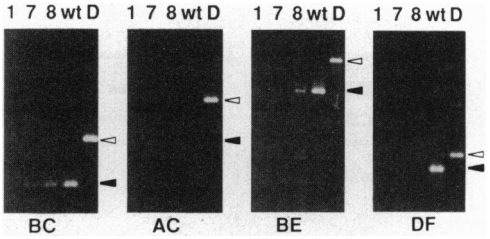

FIG. 6. RT–PCR analysis of *met2* transcription in Met⁻ derivatives of Dup1, Dup7, and Dup8. BC, AC, BE, and DF indicate the pairs of primers used (Fig. 1). Sizes of amplification products were determined after electrophoresis in 1.8% agarose gels. Lanes: D, control PCR amplification from DNA; 1, 7, 8, and wt, RT–PCR amplification from Dup1, Dup7, Dup8, and wild-type total RNAs, respectively. Expected sizes of the DNA amplification products (open arrowheads) and of the cDNA amplification products (solid arrowheads) are as follows: 588 bp and 423 bp (BC); 755 bp and no band (or 590 bp, if the transcription were initiated upstream of primer A) (AC); 1030 bp and 814 bp (BE); 515 bp and 464 bp (DF). Sizes of the cDNA amplification products were predicted with the assumption that transcripts were spliced.

Dup7 and Dup8 derivatives (Fig. 6). Wild type and one Dup1 derivative were used as positive and negative controls, respectively. The products expected from amplifications made with each of the three pairs of primers located within the transcription region (BC, BE, and DF; see Fig. 1) were observed with wild type but were not detected with the Dup1 derivative. Aliquots from the same cDNA preparation were used for amplifications with each of the four pairs of primers used, allowing a comparison between the results obtained from the different Dup derivatives and from wild type. The presence of bands with the BC pair of primers shows that the upstream part of the *met2* transcript was synthesized in Dup7 and Dup8 derivatives. The absence of bands with the AC pair of primers confirms that these transcripts were initiated in the same initiation area as in wild type. The absence of bands with the DF pair of primers in all Dup derivatives shows that transcripts did not extend to the end of the transcription region, hence confirming the data of Northern analysis. Interestingly, bands were obtained with the BE pair of primers from the Dup8 derivative, where methylation starts downstream from the segment to be amplified (Fig. 1), whereas none was obtained from the Dup7 derivative, in which methylation starts within the segment to be amplified. These results show that truncated transcripts were synthesized in Dup7 and Dup8 derivatives. The lengths of these were as expected if transcript elongation were blocked by methylation. These truncated transcripts were spliced. Southern hybridization of the amplification products using probe II of Fig. 3 (data not shown) allowed us to check their *met2* specificity. However, it allowed detection of small amounts of amplification products of the expected sizes with primers BC (with Dup1), BE (with Dup7), and DF (with Dup8). Two hypotheses may account for this observation. First, the blockage of transcript elongation by methylation could be progressive rather than sudden, allowing some transcripts to proceed through the beginning of the methylated region. Second, in some nuclei the *met2* gene could be incompletely methylated at the border of the duplicated segment, as suggested by Southern hybridization. This observation is consistent with the finding that Dup5 derivatives give apparently full-sized transcripts although duplication starts 125 and 400 bp upstream from the first and second termination sites, respectively. It is also consistent with the size of the Dup8 transcripts determined by Northern analysis, 1.2–1.5 kb, which is larger than the 1-kb size expected if transcription stopped at the boundary of the duplicated region.

DISCUSSION

Effects of Duplications on Cytosine Methylation. This work shows that duplication of a fragment of *met2* as short as 1.2 kb is sufficient to trigger MIP. Duplications involving different exogenous sequences (2, 3) or involving either the entire endogenous *met2* gene or distinct parts of it are efficient targets for MIP. This suggests that the duplication by itself constitutes the only signal directing MIP and that particular signal sequences are not required.

Our results strongly suggest that methylation extends over the length of the duplications, possibly with lower intensity close to the boundaries. However, we cannot completely rule out that it extends with a still decreasing efficiency slightly beyond the ends of the duplication, as was found for RIP-induced mutations in *N. crassa* (19). The dependence of the extent of methylation on the length of homology constitutes a further strong argument for methylation being mediated directly by the recognition between the two elements of the duplication, which probably involves a pairing step (3).

These properties of MIP now make it possible to methylate definite regions of a gene *in vivo*. This opens the way for investigating the effects of cytosine methylation upon various aspects of DNA metabolism (e.g., transcription, mutation, recombination).

Effect of DNA Methylation on Transcript Elongation. *met2* transcripts are not detected when methylation involves either the entire *met2* gene or only the region 5′ to the ORF, whereas a low amount of small transcripts is still observed when methylation begins 1.2 kb downstream from the transcription start. This shows that methylation of distinct *met2* segments has different effects upon *met2* transcription. RT–PCR analysis indicates that these small transcripts are initiated in the same region as in wild type and have the size expected if methylation blocked transcript elongation. Several observations suggest either that this blockage is progressive, transcript elongation being sometimes able to overcome stretches of several hundred nucleotides at the beginning of the methylated region, or that a fraction of the *met2* copies have lost their methylation on the border of the duplicated region. Our results also show that transcripts do not need to be full-sized to be spliced, since short transcripts have lost their intron(s). The hypothesis that DNA methylation might inhibit elongation of RNA transcripts was previously proposed from the observation that methylation of the structural portion of the herpes simplex virus thymidine kinase gene strongly inhibits its expression (20, 21). The present data constitute direct arguments for such an effect. It is important to keep in mind that generalization of our observations to other organisms such as mammals must be done with care, since methylation resulting from MIP is far more extensive than methylation in mammals, which involves essentially CpG dinucleotides.

Methylation usually represses transcription initiation by preventing essential transcription factors from interacting with the gene. Some transcription factors are directly sensitive to the methylation of their binding sites. This has been shown for the cAMP response element-binding protein (22) and for two factors that stimulate adenovirus promoters in HeLa cells (23, 24). Proteins which specifically bind to methylated CpGs can also cause transcriptional repression by excluding transcription factors (25, 26). In the present study, the possibility that methylation of the promoter area (in Dup1, Dup2, and Dup4 derivatives) also prevents transcription initiation must still be considered.

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