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**Physical mapping of the central terminator for transcription on the bacteriophage M13 genome**

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**ABSTRACT**

With the aid of in vitro transcription and translation studies it has been demonstrated that termination of transcription on bacteriophage M13 replicative form DNA occurs at a unique site which is located immediately distal to the 3'-end of gene VIII, the gene which codes for the major capsid protein. The position of this site has been mapped accurately on the enzyme cleavage maps by transcription of restriction fragments of M13 RF DNA. The central termination site was found to be located in restriction fragment Hap-B<sub>2</sub> at 450 nucleotides from the 5'-end of its viral strand (0.77 fractional length clockwise from the unique Hind II enzyme cleavage site).

**INTRODUCTION**

Transcription in vitro of the circular double-stranded replicative form DNA of the small filamentous bacteriophages (such as M13, fd and f1) with RNA polymerase from Escherichia coli results in the formation of a discrete number of RNA species ranging in size from about 8S upto 26S<sup>1,2</sup>. All RNA species contain a unique nucleotide sequence at their 5'-end<sup>3,4</sup>. Since the longest RNA, viz. 26S RNA represents nearly one round of transcription of the DNA template and the RNA polymerase has been shown to copy only the "non-viral" strand of RF DNA, it has been postulated that the different RNA classes are the result of a transcription process according to which RNA synthesis is initiated at different promoter sites but is terminated at the same unique termination signal<sup>1,5</sup>.

Such a model implies, however, that all RNA species contain identical

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Abbreviations used : RF, refers to covalently closed double-stranded replicative form DNA. Hind, Hae, Hap, and Alu refer to fragments obtained after cleavage of M13 RF DNA with the restriction endonucleases from Haemophilus influenzae (endo R. Hind II), H.aegyptius (endo R. Hae III), H.aphrophilus (endo R. Hap II) and A. luteus (endo R. Alu), respectively.

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nucleotide sequences at their 3'-terminal end. Moreover, all phage M13 mRNA species should share the coding information for a protein the gene of which is located most proximal to the central termination site. Recent in vitro protein synthesis studies, which are directed by the various M13 RNA species synthesized in vitro<sup>2</sup> as well as in vivo<sup>21</sup>, have demonstrated that all size classes of RNA are able to synthesize the protein encoded by gene VIII. From these results it is inferred here that the central termination site is located immediately distal to this gene.

As a part of our studies on the regulation of the expression of the M13 genome we are seeking to determine the DNA nucleotide sequence which signals for this transcriptional termination. Recently, the genetic map of M13 has been related to several restriction enzyme cleavage maps<sup>7-10</sup>. Consequently, DNA restriction fragments which are presumed to contain this central terminator are now available<sup>14</sup>. For this reason we have studied the transcriptional properties of these fragments in order to map, more accurately, this termination signal. Our results suggest that termination of transcription on M13 RF DNA occurs at a site which is located approximately 450 nucleotides from the left-hand side of restriction fragment Hap-B<sub>2</sub> (figs. 2 and 5).

### MATERIALS AND METHODS

Phage M13 RF DNA was prepared as described previously<sup>19</sup>. E.coli RNA polymerase holoenzyme was a generous gift of Dr. R. Schilperoort (University of Leiden). Methods for the preparation and purification of restriction fragments of M13 RF DNA have been described previously<sup>7,8</sup>.

RNA synthesis in vitro was performed in a standard reaction mixture (0.05 ml) which contained : Tris-HCl (pH 7.9), 2  $\mu$ mol; KCl, 7.5  $\mu$ mol ; MgCl<sub>2</sub>, 0.4  $\mu$ mol ; dithiothreitol, 0.05  $\mu$ mol ; EDTA, 0.05  $\mu$ mol ; Tween-80 0.1% ; bovine serum albumin, 12.5  $\mu$ g ; M13 RF-I DNA, 0.1 pmol and E.coli RNA polymerase, 1.0 pmol. After a preincubation period of 5 min at 37°C, ribonucleoside triphosphates, one of which was ( $\alpha$ -<sup>32</sup>P) labeled UTP, were added to a final concentration of 80  $\mu$ M. Initiation of RNA synthesis was allowed to proceed for one minute after which time rifampicin was added (final concentration 25 $\mu$ g/ml).

In experiments using either ( $\gamma$ -<sup>32</sup>P) labeled ATP or GTP, initiation was allowed to proceed for 3 min at 37°C. In these particular cases the concentration of the triphosphates was 6  $\mu$ M for the labeled- and 80  $\mu$ M for the non-labeled ones. Prior to the addition of rifampicin the concentration

of GTP and ATP was adjusted to 80  $\mu$ M. After the addition of rifampicin, RNA synthesis was continued for 5 min at 37°C.

Restriction fragments were transcribed under similar reaction conditions with the exception that the molar ratio of RNA polymerase to DNA template was 40.

After incubation the reaction mixtures were extracted with phenol and the RNA was precipitated twice with 2.5 volumes of ethanol at -20°C. Finally, the RNA was collected by centrifugation (15 min at 150,000 g) and dissolved in 20  $\mu$ l of pure formamide. After dissolution the RNA was analysed by electrophoresis for 4 h at 30 mA (about 150 V) on vertical slab gels (16 cm x 14 cm x 0.2 cm), containing 3.8% polyacrylamide in 98% formamide<sup>12</sup>. After electrophoresis the wet gel slab was exposed to X-ray film (Kodak, RP/R54).

For in vitro protein synthesis studies, the radioactive bands were cut from the gel and the RNA was extracted electrophoretically<sup>8</sup>. Occasionally, the RNA was further purified by CF11-cellulose chromatography as described by Franklin<sup>22</sup>. Cell-free protein synthesis, under the direction of the in vitro synthesized phage M13 mRNA species, was carried out according to the method of Konings *et. al.*<sup>11</sup>.

## RESULTS AND DISCUSSION

Transcription in vitro of bacteriophage M13 replicative form DNA by E.coli RNA polymerase holoenzyme results in the formation of at least seven RNA species, ranging in size from about 8S upto 26S. These can be separated readily by polyacrylamide gel electrophoresis (fig. 1b). Experiments performed in the presence of ( $\gamma$ -<sup>32</sup>P) labeled ribonucleoside triphosphates revealed that two of these RNA species (23S and 26S) are initiated with pppA (fig 1c ; A-start) while the other five (8S, 11S, 14S, 17S and 19S) are initiated preferentially with pppG (fig. 1d ; G-start). Furthermore, the results clearly demonstrate that the RNA species with a size of 8S, 14S and 19S are synthesized in much larger quantities than the other RNA transcripts (cf. fig. 1d).

The synthesis of a discrete number of well-defined RNA species implies that an equivalent number of promoter sites (RNA initiation sites) exist on the M13 genome. Recently, three of these promoter sites have been localized on the genetic map with the aid of coupled in vitro transcription and translation studies<sup>13,14</sup>. Furthermore, on the basis of the size of RNA chains synthesized on restriction fragments, we have been able to localize, more precisely, these and the other phage promoters on the restriction enzyme

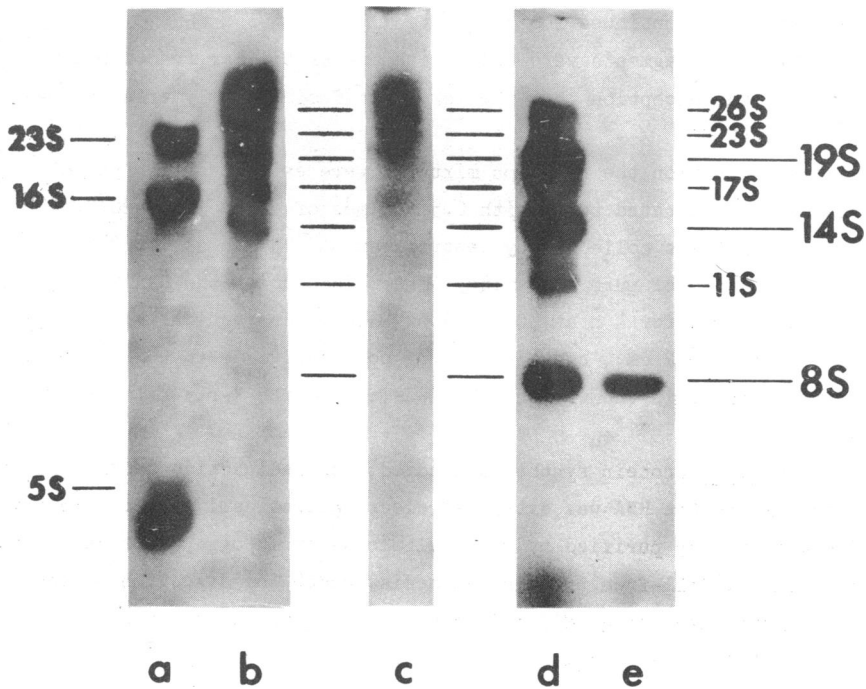


Figure 1 :

Autoradiogram of the RNA species synthesized in vitro under the direction of either M13 RF DNA or restriction fragment Hap-B<sub>2</sub>. The RNA products were analysed on 3.8% polyacrylamide slab gels containing 98% formamide (12).

(a) : E.coli ribosomal RNA markers

(b),(c) and (d) : The RNA products synthesized under the direction of M13 RF DNA in the presence of ( $\alpha$ -<sup>32</sup>P)-UTP, ( $\gamma$ -<sup>32</sup>P)-ATP and ( $\gamma$ -<sup>32</sup>P)-GTP

(e) : The RNA product synthesized under the direction of fragment Hap-B<sub>2</sub> in the presence of ( $\gamma$ -<sup>32</sup>P)-GTP

cleavage maps <sup>2</sup>. The positional pattern of M13 phage promoters detected, together with their respective initiation signals, are presented in fig. 2. A generally similar distribution of RNA polymerase binding sites along the physical maps has been reported recently by Seeburg and Schaller <sup>10</sup>.

As outlined in the Introduction in vitro protein synthesis studies directed by phage M13 specific mRNA's, which are synthesized both in vitro <sup>2</sup> as well as in vivo <sup>21</sup>, have indicated that the termination site for transcription is located immediately after gene VIII, the gene which codes for the major capsid protein <sup>6</sup>. In order to substantiate this observation, we have studied in more detail the RNA synthesis under the direction of

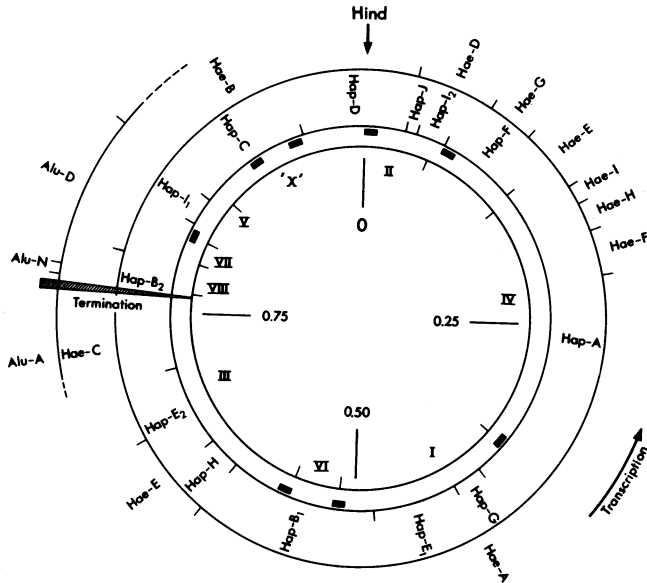


Figure 2 :

Genetic map and physical maps of bacteriophage M13.

The inner circle represents the genetic map(8). The middle and outer circles show the respective locations of the Hap II- and Hae III fragments(7). Part of the physical map, obtained after cleavage of RF DNA with the Alu enzyme, is indicated by the broken circle.

The positions of the "G-promoter" are indicated with black bars, the positions of the "A-promoter" are indicated with hatched bars. The approximate position of the central terminator for transcription is indicated.

restriction fragment Hap-B<sub>2</sub> (800 base pairs<sup>7</sup>; fig. 2). Previously, this fragment has been shown to direct, in a DNA-coupled in vitro protein synthesizing system, the synthesis of gene VIII protein<sup>14</sup>. As is demonstrated in figure 1e, fragment Hap-B<sub>2</sub> directs the in vitro synthesis of an RNA species which is approximately 360 nucleotides (8S) long. The size of this RNA is exactly the size of the smallest RNA transcribed from the intact M13 genome (fig. 1d). In addition, both RNA chains are preferentially initiated with pppG. Furthermore, as one should expect on the basis of the results published elsewhere<sup>2,14,21</sup>, both the smallest transcriptional product derived from the intact M13 genome (data not shown) and the 8S RNA species transcribed from fragment Hap-B<sub>2</sub> direct in vitro only the synthesis of the protein encoded by gene VIII (fig. 4c). Hence it is reasonably certain that both RNA transcripts are initiated and terminated at the same functional sites of the M13 genome.

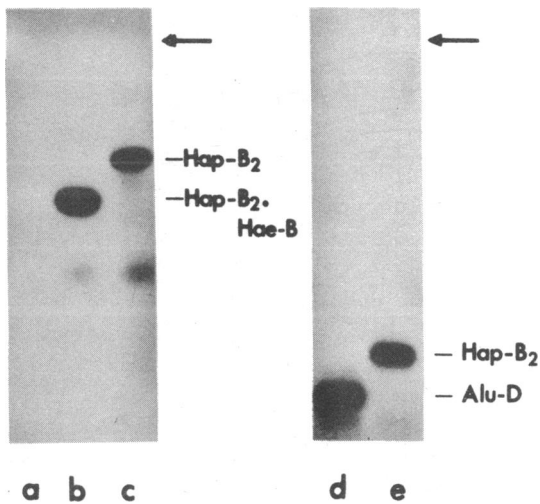
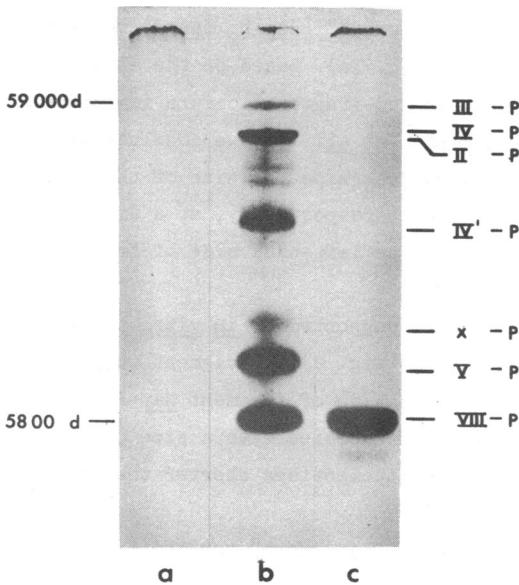


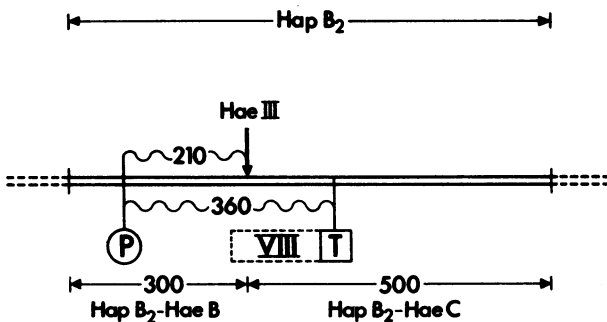
Figure 3 : Autoradiogram of the RNA species synthesized *in vitro* under the direction of (a): fragment Hap-B<sub>2</sub>·Hae-C ; (b): fragment Hap-B<sub>2</sub>·Hae-B ; (c) : fragment Hap-B<sub>2</sub> ; (d) : fragment Alu-D ; (e) : fragment Hap-B<sub>2</sub>. RNA synthesis was carried out in the presence of ( $\alpha$ -<sup>32</sup>P)-UTP. The arrow refers to the position of migration of *E.coli* 16S ribosomal RNA. Note the difference in migration distance between the 8S RNA species in both electrophoretic runs (fig. 3c and 3e).

Cleavage of restriction fragment Hap-B<sub>2</sub> with the Hae III restriction endonuclease resulted in the formation of two new fragments <sup>7</sup>, designated Hap-B<sub>2</sub>·Hae-B and Hap-B<sub>2</sub>·Hae-C, with respective lengths of approximately 300 and 500 base pairs (figs. 2 and 5). The former fragment, which is derived from the left-hand side ("5'-end") of fragment Hap-B<sub>2</sub>, can rescue amber mutations within gene VIII <sup>8</sup>. These observations suggest that the promoter which is responsible for the expression of gene VIII on fragment Hap-B<sub>2</sub> is located on this "300-fragment". This, together with the observation that the 8S mRNA is only 360 nucleotides long, suggests that the central termination site for transcription is located somewhere within the "500-fragment" (fragment Hap-B<sub>2</sub>·Hae-C).

In order to localize this termination site more precisely we have studied *in vitro* RNA synthesis both under the direction of the "300-fragment" (Hap-B<sub>2</sub>·Hae-B) as well as under the direction of the "500-fragment" (Hap-B<sub>2</sub>·Hae-C). As shown in figure 3b, fragment Hap-B<sub>2</sub>·Hae-B directs the synthesis of an RNA species which is approximately 210 nucleotides long. This size is in fact 150 nucleotides shorter than the size of the 8S RNA transcribed from



**Figure 4** : Autoradiogram of the polypeptides synthesized *in vitro* under the direction of the *in vitro* synthesized phage M13 RNA's and separated by SDS-polyacrylamide gel electrophoresis.  
 (a) : products synthesized in the absence of added RNA ; (b) : products synthesized in the presence of the mRNA's transcribed from M13 RF DNA ; (c) : products synthesized in the presence of the mRNA (8S RNA) transcribed from fragment Hap-B<sub>2</sub>



**Figure 5** : Schematic diagram of a portion of the M13 genome.  
 P and T refer to the respective positions of the promoter and termination site on fragment Hap-B<sub>2</sub>. The Hae III enzyme cleavage site on this fragment is indicated. The wavy lines refer to the RNA products transcribed either from fragment Hap-B<sub>2</sub> or from the "300-fragment". The sizes of the restriction fragments and of the RNA transcripts are indicated by arabic numerals. The roman numeral refers to the location of gene VIII on fragment Hap-B<sub>2</sub>.

fragment Hap-B<sub>2</sub> (fig. 3c). Under the reaction conditions used, no RNA synthesis could be demonstrated when the "500-fragment" (Hap-B<sub>2</sub>·Hae-C) was added to the in vitro RNA synthesizing system (fig. 3a). Based on the assumption that termination of RNA synthesis on the "300-fragment" occurs immediately proximal to its 3'-terminal end (cf. Heyden et al. <sup>20</sup>), we conclude that the biologically most important signals which determine the size of the 8S mRNA (promoter and termination site) are located, respectively, at a distance of approximately 90 and 450 nucleotides from the left-hand side of fragment Hap-B<sub>2</sub> (fig. 5).

The latter conclusion could firmly be supported by in vitro transcription studies on restriction fragment Alu-D (fig. 2), a fragment which encompasses 380 base pairs of the left-hand side of fragment Hap-B<sub>2</sub> <sup>9</sup>. In the latter case only the synthesis of an RNA species with a size of 300 nucleotides could be demonstrated, i.e. 60 nucleotides shorter than that of the 8S RNA (fig. 3d).

It should be noted that neither the "300-fragment" nor the fragment Alu-D are able to direct the in vitro synthesis of the protein encoded by gene VIII (Konings et al., unpublished results). These observations suggest that only part of gene VIII is located on these restriction fragments and, therefore, the nucleotide sequence which codes for gene VIII protein is located proximal to the 3'- rather than the 5'-end of the 8S mRNA (fig. 5).

A particular observation has to be emphasized. The size of the 8S mRNA (360 nucleotides) is about twice as large as the size of the mRNA (about 180 nucleotides) required to code for gene VIII protein (mol wt 5,800) <sup>14</sup>. Two hypotheses could account for this observation :

- 1) The 8S mRNA contains a stretch of RNA at its 5'-terminal end which does not code for protein. In this connection it is interesting to note that Sugimoto et al. <sup>15</sup> have found a long stretch of RNA which does not code for protein at the 5'-end of the RNA initiated at the phage fd promoter which is located on restriction fragment Hap-C (map position 0.94 ;fig. 2).
  - 2) The 8S mRNA contains the coding information for both the proteins encoded by gene VIII as well as by gene VII, a gene which is located on the genetic map immediately proximal to the "5'-end" of gene VIII <sup>8</sup> (fig. 2).
- At the present time there is no experimental data which would either refute or validate the first hypothesis. We favour, however, the latter hypothesis since genetic studies have indicated that all of the amber mutations within gene VII, tested so far, are rescued by fragment Hap-B<sub>2</sub> <sup>8</sup> (van den Hondel et al., unpublished results). Furthermore, indirect observations have indicated that the size of gene VII must be small i.e. 350 nucleotides or less <sup>8,18</sup>.
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Although one should expect, in this case, that the 8S RNA should also direct the in vitro synthesis of gene VII protein, the latter is absent or present at an undetectable level (fig. 4c). As a matter of fact, and for reasons still unknown, the synthesis of gene VII protein has not yet been detected in vivo<sup>16,17</sup> as well as in vitro<sup>13,14,18</sup>.

Nucleotide sequence analysis of the 8S RNA transcript might soon afford additional information which may test the validity of either alternative. In addition, the exact location of the terminator, as reported in this study, together with the availability of restriction fragments containing this site, will generate further studies in order to elucidate the nucleotide sequences involved in factor-independent termination.

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