Vol. 165, No. 1, 1989 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 30, 1989 Pages 512-518

MINUTE AMOUNTS OF RNA ARE SYNTHESIZED FROM SEVERAL REGIONS OF THE BACTERIOPHAGE Mu DNA DURING THE LYSOGENIC STATE

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Received October 13, 1989

The transcription of phage Mu DNA during the lysogenic state has been quantitatively analysed. For this purpose pulse-labelled RNA from two lysogens and from their nonlysogenic parental strains were hybridized to non-overlapping Mu DNA restriction fragments covering the whole phage genome. The data revealed that all regions of the prophage are transcribed at low rates and that phage promoters are involved in this transcription. For this study an improved assay for quantitative filter hybridization was employed. The high sensitivity and reproducibility that can be obtained with the assay make it suitable for the quantitative analysis of minute amounts of mRNA.

Little is known about the transcription of the temperate bacteriophage Mu during the lysogenic state (consult ref. 1). Two stable prophage states have been postulated: the "immune state", in which the repressor gene *c* is transcribed and the synthesized c repressor shuts off the early promoter (*Pe*), and the "nonimmune state", in which transcription from *Pe* takes place leading to *ner* expression and, consequently, to inhibition of the c repressor synthesis (2). Among the few Mu genes whose expression has been detected in the lysogenic state and which are therefore expected to be transcribed, are the genes *c* (2), *lig* (3-5), and *gin* (6-8).

In the quantitative filter hybridization technique, in which the labelled RNA to be measured is in solution and the probe DNA is immobilized on a membrane filter, the volume of the solution largely exceeds the volume of the filter matrix (9-13). This determines slow hybridization reactions that render the completion of the reaction with low concentrated RNA technically impossible. Measuring the binding before completion limits the assay sensitivity (the fraction of homologous RNA, which has not hybridized, is not detected) and its reproducibility (it is more difficult to reproduce a defined point during the reaction course than the end state of the reaction).

In a previous study (14) we analysed the Mu transcription throughout lytic development by hybridization of pulse-labelled RNA to plasmids that define Mu DNA segments covering the whole phage genome. In those experiments an assay for quantivative filter hybridization, that we derived from classical

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protocols (10, 11), was employed. We report here a similar analysis of the Mu transcription during the lysogenic state, and show that the aforementioned assay enables completion with low concentrated RNA.

MATERIALS AND METHODS

Bacterial Strains, Phages and Plasmids: The *Escherichia coli* K12 strains used were: W3110 (from J. Cairns) and its Mu *c*ts62 monolysogenic derivative 7566 (15); C600 (16) and its monolysogenic derivative C600(Mu *c*$^+$) (from A. Toussaint). The *Proteus mirabilis* strain MH3185 (MH3184/RP4::Mu *c*ts62, from M. Howe) was used to prepare Mu DNA free of variable end sequences containing *E. coli* DNA. The plasmids pKN80, pKN50, pKN13, pKN35, and pKN48 contain the non-overlapping Mu DNA fragments described in Table 1 (for details see ref.14).
Preparation of DNA and Labelled RNA: The preparations of Mu DNA, plasmid DNA and pulse-labelled [^{3}H]RNA were carried out as described previously (14).
Hybridization Assay: A hybridization mixture containing the labelled RNA in 50% formamide, 4 x SSC and phenol at 20% saturation, is prepared for a series of determinations. Per determination, 20 µl mixture are incubated at 38°C for 10 days with a 6-mm diameter nitrocellulose filter loaded with denatured DNA (plasmids are previously linearized). After incubation the filter is washed, treated with RNase and counted for radioactivity for 10 minutes. Unless otherwise indicated, DNA was in excess and hybridizations reached completion before 10 days of incubation (not shown). The RNAs from W3110 and 7566 (labelled at 28°C and 3 x 10^8 cell/ml) were hybridized to filters loaded with 0.01 pmol DNA, whereas the RNAs from C600 and C600(Mu *c*$^+$) (labelled at 37°C and 3 x 10^8 cell/ml) were hybridized to filters loaded with 0.1 pmol DNA.
Transcription Analysis: To analyse the RNA synthesized from a given template (e.g., from a Mu fragment), the binding of RNA from the lysogen and the binding of RNA from the nonlysogenic parental strain (background binding) to the filter type carrying the template, are determined (The binding is the fraction of the input radioactive RNA detected on the filter after hybridization). The difference between both values is called the "sb" (specific binding). "C" (cellular concentration of the newly synthesized template-specific RNA) is the product of the sb value and the incorporation rate of [^{3}H]uridine per lysogenic cell, and is expressed as template-specific TCA-precipitable cpm detected per lysogenic cell after 1 min incorporation. "TR" (Transcription Rate) results from dividing the C value by the size of the template (kb) and by the number of prophages present per lysogenic cell, and is expressed as template-specific TCA-precipitable cpm, detected per min, per kb of template and per prophage.

RESULTS AND DISCUSSION

Quantitative Filter Hybridization Assay. The assay used in this work derives from classical protocols (10, 11). Using high concentrated "lytic" Mu RNA we observed that the modifications introduced in the assay (reduction of the hybridization volume to 20 µl and use of a 6-mm diameter filter) lead to an acceleration of the hybridization rates (not shown). To investigate this effect with low concentrated "lysogenic" Mu RNA, the kinetics of binding of labelled RNA from the lysogen C600(Mu *c*$^+$) to filters loaded with full-length Mu DNA was studied. To minimize the background binding Mu DNA prepared in *Proteus mirabilis* was used. As shown in Fig. 1, no significant increase of binding was detected after 2-3 days of incubation and the maximum levels remained essentially constant for up 20 days. The binding value for this RNA (i.e., the fraction of input RNA that binds to the filter after completion) was

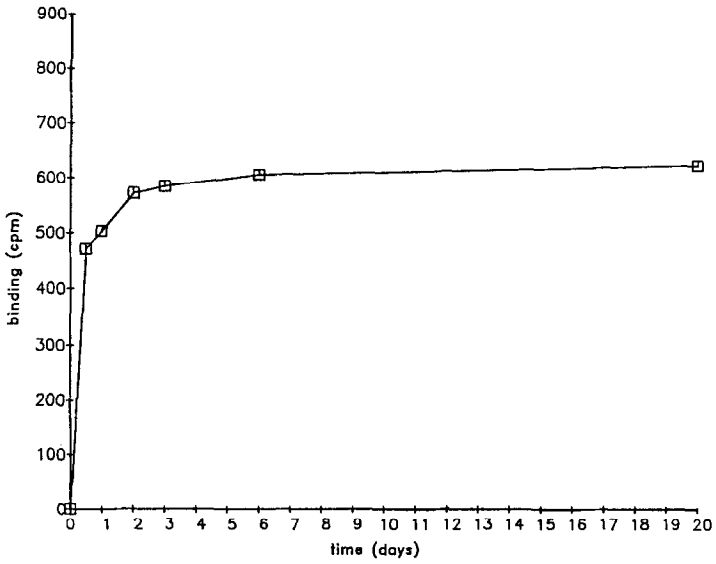

Figure 1. Kinetics of binding of C600(Mu c^+) RNA to Mu DNA filters. C600(Mu c^+) RNA was hybridized for different times with 0.1 pmol Mu DNA filters as described in Materials and Methods. The input RNA for all assays was 738,000 cpm. Each value is the average of two determinations.

(821 ± 40) x 10^{-6}, as calculated from the six values obtained at 3, 6 and 20 days. The background binding (binding of RNA from the nonlysogenic parental strain C600 measured after completion) was 74 x 10^{-6} (not shown). These data demonstrate that completion with low concentrated mRNA is technically possible and that the hybrid is quite stable. Both conditions lend the assay higher sensitivity and reproducibility (see Introduction), thus improving its ability to quantitate minute amounts of mRNA.

Currently the changes in RNA concentration are quantified with Northern, slot or dot blot hybridization by measuring the radioactivity of blot pieces containing the hybrids, or by using scanning densitometry to quantitate the signals on the blot autoradiogram. The quantitative ability can be improved by including in the blot a standard RNA. However, the reproducibility of these methods depends on parameters that often vary among different samples, RNA species and experiments (transfer efficiency of the RNA to the blot membrane, specific radioactivity of the probe DNA, state of the hybridization reaction at the incubation end, exposition time for autoradiography). Other techniques in which the parameters affecting reproducibility are more easily controllable are the quantitative hybridization in liquid and in filter. Although the hybridization in liquid works at higher reaction rates, the reannealing of the probe DNA strands limits its ability for the quantitation of low RNA concentrations. With the introduced modifications, our filter hybridization assay becomes a suitable tool for the study of gene expression at extreme low levels.

TABLE 1. Mu-specific binding (sb), Concentration of specific RNA (C) and Transcription Rate (TR) along the Mu DNA in the lysogen 7566[a]

DNA on filter	Mu DNA fragment[b] Extension (kb)	Region	Binding W3110 RNA ($x10^{6}$)	Binding 7566 RNA ($x10^{6}$)	sb[c] ($x10^{6}$)	C[c] ($x10^{8}$)	TR[c] ($x10^{8}$)
Mu DNA	0.0-37.2	*c-mom*	36	433	397	393	11
pBR322	-	-	33	34	ns[d]	ns	ns
pKN80	0.0-5.1	*c-kil*	33	110	77	76	15
pKN50	5.1-14.5	*gam-E*	32	135	103	102	11
pKN13	14.5-17.2	*E-F*	32	71	39	39	14
pKN35	17.2-23.7	*F-L*	26	79	53	52	8
pKN48	23.7-37.2	*N-mom*	43	129	86	85	6

[a] Labelled RNA from *E. coli* W3110 and from its Mu cts62 derivative 7566 were hybridized to filters loaded with 0.01 pmol of full-length Mu DNA, pBR322 and five pKN plasmids, as described in Materials and Methods. The input (radioactive RNA per hybridization assay) for W3110 RNA was 672,670 cpm and that for 7566 RNA was 646,770 cpm.

[b] A Mu restriction fragment is cloned in the pKN plasmid indicated in the first column, extends between the restriction sites whose locations are expressed as kb from the left end of the Mu genome (data from Howe [21]), and comprises the region between the given Mu genes (denoted by letters).

[c] Defined in Materials and Methods.

[d] ns=not significant.

Mu Transcription in the Lysogen 7566. Labelled RNA from the non-induced Mu lysogen 7566 (*E. coli* derivative harbouring a temperature-sensitive prophage) was hybridized to filters loaded with full-length Mu DNA, pBR322 and five pKN plasmids containig five non-overlapping Mu restriction fragments covering the whole phage genome. To minimize the background binding, the Mu DNA was prepared in *P. mirabilis*, and all plasmids were purified by three cycles of CsCl-ethidium bromide equilibrium centrifugation. We measured the background binding directly by hybridizing the RNA from the nonlysogenic parental strain W3110 to the same set of filters, and used it to subtract the whole nonspecific binding of 7566 RNA (including the binding to the *E. coli* sequences cloned in pKN80 [left Mu end] and pKN48 [right Mu end]). Conventionally the background is reduced by prehybridizing the filter with heterologous DNA. For the quantitation of minute amounts of RNA we consider our approach more appropriate than the prehybridization step, because a small degree of homology between the heterologous DNA and the sequences sought for, that can never be excluded, could lead to a partial loss of the scarce specific signals.

Table 1 shows the binding data for 7566 and W3110, and the values for the Mu-specific binding "sb", Concentration of specific RNA "C" and Transcription Rate "TR". As expected, the background values (binding of W3110 RNA) were low and constant, and were always lower than the binding of 7566 RNA. In addition, the sum of the sb for the five Mu fragments (358×10^{-6}) coincided well with the sb for the full-length Mu DNA (397×10^{-6}). Under these conditions the sb, C and TR values exclusively reflect transcription from Mu DNA sequences and

TABLE 2. sb, C and TR along the Mu DNA in C600(Mu c^+)[a]

DNA on filter	Mu DNA fragment	sb ($x10^6$)	C ($x10^8$)	TR ($x10^8$)
pKN80	*c-kil*	148	240	47
pKN50	*gam-E*	203	329	35
pKN13	*E-F*	150	243	90
pKN35	*F-L*	167	271	42
pKN48	*M-mom*	247	400	29

[a] Labelled RNA from C600 and C600(Mu c^+) were hybridized to 0.1 pmol DNA filters. sb, C and TR were calculated as indicated in Materials and Methods.

indicate that all fragments are transcribed at low rates during the lysogenic state. The TR values revealed a slightly higher activity in the leftmost and central fragments (cloned in pKN80 and pKN13, respectively).

Mu Transcription from Spontaneously Induced Lysogens. To evaluate the contribution of the spontaneously induced lysogens to the Mu transcription levels measured in the non-induced 7566 culture, we employed the parameter C expressing the concentration of newly synthesized fragment-specific RNA in a lysogenic cell. First, we calculated for every Mu fragment the average of the C values registered at 2, 4, 5.5, 7, 9, 11, 14, 17, 20, 23, 26, 31, 41 and 46 min after the heat induction of a 7566 culture (We reported these values previously [14]; the labelling and hybridization conditions for these lytic RNAs were identical with those for the lysogenic 7566 RNA); this average well represents the C of the fragment-specific RNA in a hypothetical population of *asynchronously* induced 7566 lysogens. By further multiplication with 10^{-4} (frequency of spontaneous induction in the non-induced culture) we obtained a value representing the C of the fragment-specific RNA in the *subpopulation of spontaneously and asynchronously* induced lysogens, which is present in a non-induced 7566 culture. The resulting C values for the five Mu fragment-specific RNAs (not shown) were always less than 1% of the corresponding C values in the non-induced 7566 (shown in Table 1), indicating that the contribution of the spontaneously induced lysogens is not significant.

Mu Transcription in the Lysogen C600(Mu c^+). We also hybridized RNAs from C600(Mu c^+) and C600 to the same set of pKN plasmids. As the filters used with these RNAs contained 10-fold more probe DNA than those used with 7566 RNA and W3110 RNA, higher binding efficiencies, and thereby higher sb values should be expected. The labelling temperature for C600(Mu c^+) and C600 was 9°C higher than that for 7566 and W3110; this should increase the incorporation rate of uridine, and consequently, the C and TR values. As expected, all the sb, C and TR values for C600(Mu c^+) (Table 2) were higher than those for 7566 (Table 1). However, as in 7566, the TR values revealed transcription from all prophage regions with a relative preference for the leftmost and central fragments.

Involved Promoters. The only bacterial promoter that could be involved in the lysogenic Mu transcription is that of the gene in which the prophage is inserted. As *E. coli* genes are transcribed unidirectionally (17), any extension of host transcription would enter into the prophage DNA only from one Mu end, continuing onto the Mu strand to which the host coding strand is covalently linked. The 5' end of the host coding strand can be linked, either to the 3' end of the Mu *r* strand or to the 3' end of the complementary Mu *l* strand; in both cases the linked Mu strand and thereby its coded trancription units (the *r* strand codes for the lytic transcription units and the *l* strand for the gene *c* [15, 18, 19]) will be transcribed only in the codogenic sense.
From these considerations it can be inferred that:

- If the RNA polymerase enters into the *r* strand (only possible from the left Mu end), it should recognize the termination signals of the lytic transcription units. This should be reflected by a pattern consisting of a gradient of the TR values ranging from the leftmost Mu fragment to the rightmost one.
- If the polymerase enters into the *l* strand (only possible from the right Mu end), it could transcribe almost the entire Mu genome until the stop signal of gene *c* located near the left Mu end. The resulting pattern should show either a single TR value for all fragments (if transcription proceeds without interruptions), or a gradient of the TR values ranging from the rightmost Mu fragment to the leftmost one (if interruptions at random take place).

As neither of the analysed prophages showed the patterns predicted above, we infer that transcription from host promoters cannot be *exclusively* responsible for the observed patterns, and that Mu promoters must be involved. The finding that two lysogens containing prophages in different positions of the bacterial chromosome showed similar patterns strongly suggests that the Mu transcription is independent of the insertion site and is therefore *mainly* or even *exclusively* directed from phage promoters. Among these are, certainly those that direct the transcription of *c*, *lig* and *gin* (genes expressed in lysogenic cells), and probably *Pe* (active in the nonimmune prophage state). Finally, every Mu promoter active during the lytical development could contribute with a remaining background activity. In this sense, the promoter for the late operon comprising the genes *D*, *E*, *H* and *F* (14, 20) is probably mainly responsible for the relative higher TR levels of the central fragment (*E-F*).

ACKNOWLEDGMENTS

C. Barron was a fellow of the Deutscher Akademischer Austauschdienst, Bonn-Bad Godesberg, FRG. Supported by the Deutsche Forschungsgemeinschaft (Ba 600/6-4).

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