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Quantification of polymerase chain reaction products by affinity-based hybrid collection

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ABSTRACT

We have used oligonucleotides modified with biotin in the 5'-end as primers in the polymerase chain reaction (PCR)-amplification. This results in the synthesis of 5'-biotinylated DNA molecules, which are detected by hybridization to a labelled probe in solution. The formed hybrids are collected on an avidin-matrix by mediation of the biotin residue of the target molecules. The affinity-based hybrid collection method is quantitative and makes it possible to measure the amount of DNA produced in the PCR-amplification. At low concentrations of template the efficiency of the process is close to 100 %, making it possible to detect the presence of a few molecules of target DNA in 25 cycles. With high template concentrations the efficiency of the process is low.

INTRODUCTION

Nucleic acid hybridization has become a powerful tool in viral diagnosis due to its high specificity. However, for many applications the sensitivity of the tests is not high enough without enrichment of the microbe. For identification of cytomegalovirus (CMV) the sensitivity of the present hybridization methods is sufficient for the analysis of urine samples from newborns, but not enough for a more general use (1,2).

A promising approach to solve the sensitivity problem in hybridization assays is to amplify the amount of nucleic acids in a sample *in vitro* before detection by hybridization. This can be done by the polymerase chain reaction (PCR) using specific oligonucleotide primers and DNA-polymerase (3). In the PCR-amplification repeated cycles of denaturation of the template, primer annealing, and extension of the primers results in an exponential increase in the number of template molecules. Theoretically a million-fold amplification is achieved in 20 cycles. The use of a

heat stable DNA-polymerase isolated from the Thermus aquaticus bacterium simplifies the procedure and has made its automatization possible (4).

The PCR-amplified DNA-sequences are usually detected by conventional spot- or Southern blot hybridization (5-7). Methods involving restriction enzyme cleavage of the amplified products have also been applied (8,9).

The affinity-based hybrid collection method developed in our laboratory is more convenient for the detection of nucleic acids than the techniques mentioned above. In this method the hybridization reaction is performed in solution, and the formed hybrids are captured on an affinity-matrix with the aid of a probe modified with an affinity label (e.g. biotin) (10).

Our aim was to create a sensitive and convenient diagnostic test by combining the PCR-amplification with the affinity-based hybrid collection procedure.

MATERIALS AND METHODS

Cytomegalovirus DNA-reagents

The CMV template used was a fragment of the long unique region of the CMV genome at map position 0.30-0.35, i.e. the 12.2 kb Hind III L-fragment of AD 169 CMV DNA cloned in the pAT153 vector (1,11). The plasmid was linearized with Eco RI. The region of CMV DNA, which was amplified, spans the junction between two adjacent Bam HI fragments (4.2 and 2.5 kb) of CMV Hind III L (1). The nucleotide sequence of the Bam HI junction was determined (12) to enable the synthesis of the PCR-primers and a detector probe.

The oligonucleotide primers and probe were synthesized on an Applied Biosystems 381A DNA synthesizer by the β-cyanoethyl phosphoramidite method (13). The PCR-primers (denoted 35A and 35B) were 35-mer oligonucleotides flanking a 115 bp region in the CMV Hind III L-fragment (see Figure 2). A 5'-terminal aminogroup was added as the last step in the synthesis of the PCR-primers using the aminolink II (P/N 400808, Applied Biosystems).

A 70-mer oligonucleotide complementary to the amplified sequence was used as detector probe. It was labelled by primer extension from a 20-mer primer (see Figure 2).

A 110 bp Hae III fragment corresponding to the amplified sequence was cloned in M13 mp 10 in both orientations. These were biotinylated and used as quantification standards in the affinity-based hybrid collection method.

Biotinylation

Biotin was introduced into the linearized CMV-plasmid and into the M13 phages with photobiotin (Vector Laboratories Inc.) (14). The 5'-aminogroup of the PCR-primers were biotinylated with sulfo-NHS-biotin (Pierce Chemical Co.) (15). The biotinylated oligonucleotides were purified by HPLC on a reversed phase C-18 column (Bengtström et al. to be published).

Labelling with ^{32}P

The CMV-plasmid was labelled with [α-^{32}P]dCTP (Amersham,) by nick-translation (16). The 70-mer detector probe was labelled with [α-^{32}P]dCTP by primer extension from a 20-mer primer. A labelling reaction consisted of 3 pmol of 70-mer, 30 pmol of 20-mer, 66 pmol of [α-^{32}P]dCTP (>3000 Ci/mmole) and 200 μM of dATP, dGTP and dTTP, 4 units of E.coli DNA polymerase I (the Klenow fragment; Boehringer-Mannheim GmbH) in 50 μl of 50 mM tris-HCl, pH 8, 50 mM KCl, 10 mM $MgCl_2$ and 10 mM dithiothreitol. The 20-mer primer was hybridized to the 70-mer template for 15 min. at 65°C, the enzyme was added and the extension reaction was carried out for 30 min. at 15°C, after which the labelled material was purified on a Sephadex G-25 spin column. Specific activities of 4-8 x 10^7 cpm/pmol of 70-mer were obtained.

PCR-amplification

The PCR-amplification was carried out with 100 pmol (1 μM) each of the primers, 200 μM each of dATP, dCTP, dGTP and dTTP in 100 μl of a solution of 10 mM Tris-HCl, pH 8.35, 50 mM KCl, 1.5 mM $MgCl_2$ and 0.1 mg/ml gelatin in 0.5 ml Eppendorf-tubes under a layer of viscous paraffin (7160, Merck). The template DNA was denatured by boiling for 5 min. in the reaction mixture. 2 units of Thermus aquaticus (Taq) DNA-polymerase (New England Biolabs) were added, and the tubes were transferred manually between three heat blocks for 10-25 cycles as follows: Primer annealing in a 55°C heat block for 2 min., extension of the primers in a 72°C block for 3 min., and denaturation of the template in a 98°C block for 1 min. 15 sec.

Affinity-based hybrid collection

One tenth (10μl) of the PCR-mixture was directly analyzed by the affinity-based hybrid collection procedure. The hybridization solution (50 μl) contained 0.6 M NaCl, 20 mM Na-phosphate, pH 7.5, 1 mM EDTA, 0.02% Ficoll^{R}, polyvinylpyrrolidone and bovine serum albumin and about 0.02 pmol of the ^{32}P-labelled 70-mer probe. The hybridization reaction was carried out at 65°C for 1h. For quantification of the amplified biotinylated DNA a standard curve was prepared using varying amounts of the biotinylated M13 clones.

After the hybridization reaction 20 μl of a 5% (w/v) suspension of avidin-coated polystyrene particles (0.8 μM, Pandex Laboratories Inc.) were added. The capturing reaction was for 30 min. at 20°C. The particles were collected by centrifugation and washed once with 1 ml of 15 mM NaCl, 1.5 mM Na-citrate, 0.2% sodium dodecyl sulphate for 5 min. at 37°C, and twice for 1 min. at 50°C followed by 5 min. at 37°C. The radioactivity of the hybrids bound to the particles was measured.

RESULTS AND DISCUSSION

Principle of the method

We have devised a method based on the affinity-capture principle (10) with the aid of which the polymerase chain reaction (PCR) can be conveniently followed. Oligonucleotides with biotinylated 5'-ends are used as primers in the PCR. This leads to the production of nucleotide sequences carrying a biotin moiety in the 5'-end. For detection the amplified biotinylated sequences are allowed to hybridize in solution to a labelled probe, after which the formed hybrids are collected on an avidin- affinity matrix for quantification (Figure 1). In this study we used 35-mer oligonucleotides flanking a 115 bp region in the long unique region of the CMV genome as primers, which results in the amplification of a 185 bp CMV DNA-fragment. The probe was a ^{32}P-labelled 70-mer oligonucleotide complementary to the amplfied DNA fragment. The locations and the nucleotide sequences of the primers and the probe are shown in Figure 2.

Quantification of the PCR-amplification

A series of samples containing varying amounts of CMV temp-

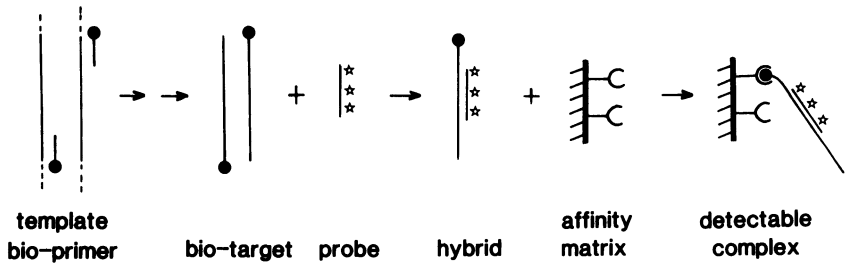
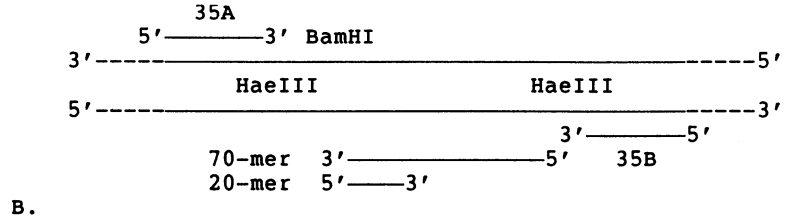


Figure 1.
Affinity-based collection of amplified biotinylated targets. PCR-amplification with one or both primers 5'-biotinylated (bio-) results in the production of biotinylated DNA fragments. After hybridization to a labelled probe the formed hybrids are captured on an avidin-affinity matrix and measured.

late (300 - 3 x 10^7 molecules) were processed through the PCR-reactions for 10, 15, 20, and 25 cycles. The amount of amplified sequences created were then quantified by hybridization in solution to the radiolabelled 70-mer probe, followed by collection of

A.



B.

35A: 5'GGT GCA CGT GTC GCA AGC TCT TTC CCG GCC TGG CT

35B: 5'ACT GTT TGG AGC TCT GCG CGA ACA TGT AGT CGG CC

70-mer: 5'GGT TGC TCT TGC TGA GCT GCA TGA GCA GCG CCG CCG
CCG TCG CCA CCT CCG GCG CTG TCC TCG CGA CGT G

20-mer: 5'ACG TCG CGA GGA CAG CGC CG

Figure 2.
A. Position on the amplified 185 bp fragment of the PCR-primers (35A, 35B), the 70-mer detector probe, and the 20-mer primer used for labelling the detector probe. The Hae III fragment used as quantitative standard and the Bam HI junction (see "Materials and Methods") are indicated. The drawing is in scale.
B. Nucleotide sequence of the primers and probe.

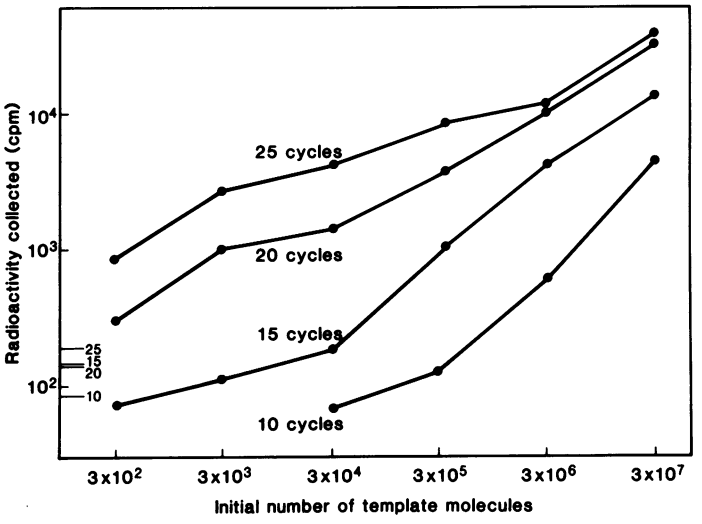


Figure 3.
Detection of biotinylated DNA synthesized in the polymerase chain reaction. Varying amounts of CMV-DNA template were amplified for 10, 15, 20 and 25 cycles. One tenth of the PCR-mixture was hybridized to 177000 cpm of the ^{32}P-labelled 70-mer probe and the radioactivity in the hybrids collected is shown (mean of duplicate amplifications). The cpm-values from control amplifications without added template are indicated with horisontal bars. The background from the probe alone (111 cpm) has been subtracted from the given values.

the hybrids on avidin-polystyrene particles and measurement of the bound radioactivity. The results of such an experiment are shown in Figure 3. In this experiment the sample containing 300 molecules of template in a 100 μl reaction gave 860 cpm after 25 cycles, while 190 cpm were bound in the corresponding control reaction without added template. Since only 10 μl of the reaction mixture was analyzed, 30 molecules of initial template were detected.

We have noted that the efficiency of the amplification reaction is strongly dependent on the quality of the Taq-polymerase used. Consequently the results, as cpm in hybrid, vary from enzyme batch to batch. Within one experiment the results are, however, consistent, and the detection limit of at least 30 molecules is reproducible.

The amount of DNA sequences synthesized was quantified with the aid of a hybridization standard curve prepared in parallel using known amounts of biotinylated DNA (Table 1). From these values the mean efficiency at different stages of the PCR-cycles was calculated (Table 2). The efficiency of the PCR varies, being very efficient at low initial template concentration and during the first 10 cycles. On the contrary, with a high template input the efficiency of the last cycles (20 - 25) is low. This behaviour makes the quantitative measurement of the original concentration of DNA difficult. If this is desired, samples should be withdrawn and analyzed at several stages of the amplification process. An internal standard may also be useful (17).

The reactions of the procedure

In order to explain the observed behaviour of the PCR-amplification we investigated the individual reactions using the affinity-capture methodology (10). The procedure described here consists of the reactions taking place during the PCR-amplification (denaturation and reannealing of the template DNA, annealing of the primers to the template, extension of the primers), the hybridization reaction between the detector probe and the amplified DNA-fragments and, finally, the capturing reaction between the biotin and the avidin-matrix.

a) Is the primer annealing quantitative?

To ascertain this, we used biotinylated oligonucleotide primers and a ^{32}P-labelled template DNA. The template was denatured and the oligonucleotides were allowed to anneal as in the PCR-reaction. The samples were immediately cooled, whereafter the formed hybrid molecules were captured on avidin-polystyrene particles. The amount of bound radioactivity is a measure of the primer annealing reaction. It was seen that when present at large molar excess (10^6-fold) the hybridization reaction between primers and template was completed in 1 min. after transfer from the denaturation temperature to the 55°C heat block (Table 3). The molar excess of primer over template can be up to 10^{11}-fold in the beginning of the PCR-process and decreases to about 100-fold in the final cycles when template molecules have accumulated. It can be calculated that at DNA concentrations typical during the

Table 1. The amount of DNA sythesized by the PCR-amplification.

Initial	Number of DNA molecules After 10 cycles	15 cycles	20 cycles	25 cycles
3×10^{7}	1.5×10^{9}	4.0×10^{9}	1.0×10^{10}	1.3×10^{10}
3×10^{6}	2.0×10^{8}	1.1×10^{9}	2.7×10^{9}	3.2×10^{9}
3×10^{5}	2.5×10^{7}	2.3×10^{8}	9.5×10^{8}	2.6×10^{9}
3×10^{4}	Nd	3.2×10^{7}	3.1×10^{8}	1.0×10^{9}
3×10^{3}	Nd	Nd	2.4×10^{8}	8.5×10^{8}
3×10^{2}	Nd	Nd	5.6×10^{7}	1.8×10^{8}

The amount of DNA produced after 10, 15, 20 and 25 cycles was determined from the cpm-values presented in Figure 3 with the aid of a hybridization standard curve. Nd = Not detectable, i.e. less than 7×10^{6} molecules.

final PCR cycles (10^{10}-10^{11} molecules/100 μl) significant reannealing of the template can be expected (18). This may reduce the primer annealing reaction and consequently the efficiency of the later PCR-cycles.

b) Is the denaturation of the template complete?

Biotinylated template molecules were incubated as for de-

Table 2. Efficiency of the PCR-cycles.

Initial amount of template (no. of molecules)	Mean efficiency (%) Cycle number 1-10	11-15	16-20	21-25	1-25
3×10^{7}	86	22	20	5.3	40
3×10^{6}	92	41	19	3.7	45
3×10^{5}	96	56	33	22	57
3×10^{4}	-	86 [1]	57	26	66
3×10^{3}	-	-	97 [2]	28	81
3×10^{2}	-	-	106 [2]	26	87

The efficiencies at different intervals of the PCR-process were calculated from the measured amounts of synthesized DNA (see Table 1) according to the formula (3): $\sqrt[n]{\text{amplification level}}-1$; n = number of cycles. 1) Mean efficiency of cycles 1-15, 2) Mean efficiency of cycles 1-20.

Table 3. Rate of the primer-annealing reaction.

Hybridization time (min)	Radioactivity in hybrids (cpm)[1)]	% of maximum
0	303	16
1	1849	97
3	1879	99
5	1891	99
15	1903	100

Biotinylated 35-mer primers (6 x 10^{13} molecules, 1 μM) were allowed to anneal to 5 x 10^{7} molecules (1 ng) of ^{32}P-labelled CMV-plasmid in the PCR-buffer at 55°C for the indicated times immediately after previous incubation at 98°C. The tubes were quickly cooled to 0°C and the formed hybrids were captured on avidin-polystyrene particles at 0°C for 30 min. The particles were washed three times with 150 mM NaCl, 15 mM Na-citrate, 1% SDS at 20°C and the bound radioactivity was measured. The cpm-value from background controls without primer (218 cpm) has been subtracted from the given values. 1) Mean value of triplicate assays.

naturation during the amplification process. The samples were cooled to 0°C and the amount of denatured template was measured by hybridization to a radiolabelled probe. The formed hybrids were collected and measured. The bound radioactivity reflects the proportion of single-stranded template initially available for hybridization. This experiment showed that the required denaturation temperature depends on the size of the template molecules (Table 4). Denaturation of the linearized 14 kb CMV-template required 93°C, while fragments smaller than 3 kb were denatured at 91°C. The denaturation temperature for small fragments is obviously also dependent on the nucleotide sequence.

c) Is inactivation of the Taq polymerase reducing the efficiency of the amplification?

Two different experiments were performed to answer this question. In one set the inactivation was measured directly. The Taq-polymerase in PCR-buffer was passed through a number of cycles. Thereafter template, biotinylated primers and dNTPs, including ^{32}P- dCTP, were added, and the extension reaction was allowed to proceed for 30 minutes. The extension product, which consequently became 5'-biotinylated and radioactive, was then collected on avidin-polystyrene particles and the bound radioactivity was measured. It was seen that the enzyme lost activity steadily, but still retained about 30 % of it after 15 cycles. In the other

Table 4. Denaturation of the template molecules.

Time (sec.)	Temperature [1]	Radioactivity in hybrids (%) Template size	
		14 kb	< 3 kb
60	91.2 °C	36	97
75	92.9 °C	99	113
90	93.9 °C	104	-
120	95.5 °C	113	-
300	97.2 °C	100	100 [2]
No denaturation		3	4

3×10^9 molecules (60 ng) of biotinylated linearized CMV-plasmid or of a biotinylated SacI-SacII digest of this plasmid (100 bp - 3 kb fragments) were heated in the PCR-buffer in a 98°C heat block as indicated. The samples were quickly cooled to 0°C. Denatured ^{32}P-labelled CMV-plasmid (150000 cpm; 2×10^7 molecules) was added. The hybridization and capture of the hybrids was as described in "Materials and Methods". 1) The temperature given is that of the solution in the test tube at the end of the incubation period. 2) The radioactivity of the collected hybrids was compared with that of a control assay, in which the denaturation was for 5 min. at 100°C.

set of experiments we investigated to what extent the addition of polymerase to the 12:th cycle of 25 improved the efficiency. In this case the effect was marginal. It should be noted that, as shown in Table 2, with low template input the mean efficiency of 25 cycles is more than 85 % and the efficiency is acceptable even at cycles 20 - 25. The enzyme:template ratio decreases continuously during the amplification process. The main reason for this is the increase in template concentration and we conclude that the decrease in enzymatic activity is not critically affecting the process.

d) Is one biotin moiety sufficient for capture?

To ensure that one biotin moiety in the 5'-end of the template molecule is sufficient for complete capture on the avidin-polystyrene particles the following experiment was carried out. Denatured CMV-plasmid was hybridized to 5'-biotinylated oligonucleotide primers. These were then elongated with DNA polymerase in the presence of ^{32}P-dCTP. The amount of radioactive extension product and the amount of product collectable on the avidin-poly-

Table 5. Capture on avidin-polystyrene particles of DNA by mediation of a single 5'-biotin residue

Primer	Radioactivity (cpm) [1] incorporated	captured	% captured
Bio-35A	685000	680000	99
Bio-35B	815000	805000	98
35A	765000	423	0.06

10^9 molecules (18 ng) of denatured CMV-plasmid was labelled with [α-^{32}P]dCTP (33 pmol) by primer extension with DNA polymerase (the Klenow fragment), using 100 pmol (1 μM) of biotinylated primer (or unbiotinylated primer as control) and 200 μM dATP, dGTP, dTTP at 37°C for 30 min. After gel filtration on a Bio-gel P-30 spin-column the labelled product was collected on avidin-polystyrene particles for 1h at 20°C. 1) Radioactivity in one tenth of the reaction mixture; two parallell assays.

styrene beads was measured. As can be seen from Table 5, complete capture of the labelled product by mediation of the single biotin residue was achieved.

The biotin-binding capacity of the amount of avidin-polystyrene particles used (20 μl of a 5% (w/v) suspension) was shown to be about 2 nmol of 5'-biotinylated oligonucleotide (data not shown), while we used 100 pmol biotinylated primer per assay.

We conclude that the affinity-based hybrid collection method is convenient for the quantification of the amount of DNA synthesized by the polymerase chain reaction. The PCR in itself is, however, not a quantitative method. This seems to be due to factors involved in the basic principle of the process, in which the growing concentration of template leads to increasing reannealing rates and decreasing enzyme:template ratios. By carefully controlling the system it is possible to obtain a semiquantitative measure of the initial amount of template DNA.

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REFERENCES

1. Virtanen, M., Syvänen, A-C., Oram, J., Söderlund, H. & Ranki, M. (1984) J. Clin. Microbiol. 20, 1083-1088.
2. Augustin, S., Popow-Kraupp, T., Heinz, F., & Kunz, C. (1987) J. Clin. Microbiol. 25, 1973-1977.
3. Mullis, K.B. & Faloona, F.A. (1987) Methods Enzymol. 155, 335-350.
4. Saiki, R.K., Gelfand, D.H., Stoffel, S., Scharf, S.J., Higuchi, R., Horn, G.T., Mullis, K.B. & Erlich, H.A. (1988) Science 239, 487-491.
5. Shibata, D.K., Arnheim, N. & Martin, W.J. (1988) J. Exp. Med. 167, 225-230.
6. Loche, M. & Mach, B. (1988) Lancet ii, 418-421.
7. Murakawa, G.J., Zaia, J.A., Spallone, P.A., Stephens, D.A., Kaplan, B.E., Wallace, R.B. & Rossi, J.J. (1988) DNA 7, 287-295.
8. Kwok, S., Mack, D.H., Mullis, K.B., Poiesz, B., Erlich, G., Blair, D., Friedman-Kien, A. & Sninsky, J.J. (1987) J. Virol. 61, 1690-1694.
9. Ou, C-Y., Kwok, S., Mitchell, S.W., Mack, D.H., Sninsky, J.J., Krebs, J.W., Feorino, P., Warfield, D. & Schochetman, G. (1988) Science 239, 295-297.
10. Syvänen, A-C., Laaksonen, M. & Söderlund, H. (1986) Nucleic Acids. Res. 14, 5037-5048.
11. Oram, J.D., Downing, R.G., Akrigg, A., Dollery, A.A., Duggleby, C.J., Wilkinson, W.G. & Greenaway, P.J. (1982) J. Gen. Virol. 59, 111-129.
12. Sanger, F., Nicklen, S. & Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463-5467.
13. Beaucage, S.L. & Caruthers, M.H. (1982) Tetrahedron Lett. 22, 1859-1862.
14. Forster, A.C., McInnes, J.L., Skingle, D.C. & Symons, R.H. (1985) Nucleic Acids Res. 13, 745-761.
15. Updyke, T.V. & Nicolson, G.L. (1986) Methods Enzymol. 121, 717-725.
16. Rigby, P.W.J., Dieckmann, M., Rhodes, C. & Berg, P. (1977) J. Mol. Biol. 113, 237-251.
17. Chelly, J., Kaplan, J-C., Maire, P., Gautron, S. & Kahn, A. (1988) Nature 333, 858-860.
18. Britten, R.J. & Kohne, D.E. (1968) Science 161, 529-540.