Digestion of highly modified bacteriophage DNA by restriction endonucleases

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ABSTRACT

The ability of thirty Type II restriction endonucleases to cleave five different types of highly modified DNA has been examined. The DNA substrates were derived from relatively large bacteriophage genomes which contain all or most of the cytosine or thymine residues substituted at the 5-position. These substituents were a proton (PBS1 DNA), a hydroxymethyl group (SP01 DNA), a methyl group (XP12 DNA), a glucosylated hydroxymethyl group (T4 DNA), or a phosphoglucuronated, glucosylated 4,5-dihydroxypentyl group (SP15 DNA). Although PBS1 DNA and SP01 DNA were digested by most of the enzymes, they were cleaved much more slowly than was normal DNA by many of them. 5-Methylcytosine-rich XP12 DNA and the multiply modified T4 and SP15 DNAs were resistant to most of these endonucleases. The only enzyme that cleaved all five of these DNAs was TaqI, which fragmented them extensively.

INTRODUCTION

Restriction endonucleases will hydrolyze a DNA molecule only if the oligonucleotide sequence which the enzyme recognizes is present (1,2). Generally, methylation of one specific cytosine (C) or adenine (A) residue within the recognition sequence will prevent cleavage at the sequence by a Type II restriction endonuclease (1,3). However, in several cases it has been shown that methylation of a different C or A residue at the recognition site does not affect digestion (4-7).

In this study we have examined the ability of thirty restriction endonucleases to digest various types of unusually modified DNAs. These DNAs are of bacteriophage origin and contain most or all of the C or thymine (T) residues replaced by a derivative modified at the 5-position. Two of these, Xanthomonas oryzae phage XP12 and Escherichia coli phage T4 DNAs, contain all of the C residues replaced by 5-methylcytosine (m^5C ; 8-10) or 5-hydroxymethylcytosine (hm^5C ; 11), respectively. The genomes of Bacillus subtilis phages SP01 and PBS1 have complete substitution of T residues with 5-hydroxymethyluracil (hm^5U ; 12) or uracil (U; 13) residues, respectively. Sixty

two percent of the T residues in the DNA of Bacillus subtilis phage SP15 are replaced by phosphoglucuronated and glucosylated 5-(4',5'-dihydroxypentyl)uracil(hp⁵U; 14,15). Given the relatively large size of these phage genomes (3×10^7 - 2.5×10^8 d; 16) and the many enzymes tested, we were able to draw conclusions about the general effects of these modifications on the ability of restriction endonucleases to cleave at their recognition sites.

MATERIALS AND METHODS

Enzymes. BstNI and MspI were obtained from New England Biolabs (Beverly, Mass.). All other restriction enzymes were from Bethesda Research Labs (Rockville, Maryland). Digestions were carried out under standard conditions as per the manufacturer's directions. One unit of restriction enzyme is defined as the minimum amount able to completely digest 1 μ g of phage λ DNA in 1 h.

Hydrolysis of DNA (25 to 40 μ g/ml) was also performed with 0.2 to 0.5 units/ml of DNase I (EP grade; Sigma, St. Louis, Missouri) in 0.05 M sodium acetate, 5×10^{-3} M MgSO₄, pH 5, or with 0.4 units/ml of snake venom phosphodiesterase (Worthington, Freehold, New Jersey) in 0.1 M Tris-HCl, 5×10^{-3} M MgSO₄, pH 8.9 at 30°C. For the latter hydrolyses, DNA samples were first heat-denatured in 1 mM Tris-HCl, 0.1 mM EDTA, pH 7.4, and 100°C for 5 min followed by rapid cooling in ice.

Substrates. Phage DNA was isolated as described previously (9) from phage purified by isopycnic CsCl gradient centrifugation. Plasmid DNA, pBR322 (17) was obtained after chloramphenicol-induced amplification (18) by brief boiling and removal of most genomic DNA (19) followed by centrifugation in gradients of CsCl-ethidium bromide (20). Bacterial DNA was extracted by standard techniques (21) and calf thymus and salmon sperm DNAs were obtained commercially (Worthington or Sigma).

Analysis of enzymatic digests. Restriction enzyme digests were analyzed by agarose gel electrophoresis and visualized by ethidium bromide-induced fluorescence as previously described (23). Hydrolysis by DNase I and venom phosphodiesterase was monitored by increases in absorption of the reaction mixture at 260 nm and the linear portion of the reaction curves was used to calculate reaction rates.

RESULTSDigestion of XP12, T4, and SP15 DNAs with restriction endonucleases.

Tables 1 and 2 show the susceptibility of five different highly modified phage DNAs and one normal phage DNA, λ DNA, to digestion by many restriction endonucleases. Lambda DNA is known to be digested by all of these enzymes and is cleaved many times by most of them (1). We checked the extent

Table 1. Digestion of highly modified DNA with restriction endonucleases with recognition sites containing four base pairs.

Type of restriction endonuclease	Recognition sequence	DNA with modified C		DNA with modified T			Normal DNA (control) λ
		residues XP12	T4	residues SP15	SP01	PBS1	
<u>AluI</u>	AGCT	-	-	-	S	S	+
<u>CfoI</u>	GCGC	-	-	-	+	+	
<u>HhaI</u>	*GCGC	-	-	-	+	+	+
<u>DdeI</u>	CTNAG	-	-	S ¹	S	+	+
<u>HaeIII</u>	*GGCC	-	-	-	+	S ¹	+
<u>HinfI</u>	GANTC	S	-	-	S	S	+
<u>HpaII</u>	*CCGG	-	-	-	+	+	
<u>MspI</u>	*CCGG	-	-	-	+	+	+
<u>MboI</u>	*GATC	S	-	-	+	+	+
<u>Sau3A</u>	GATC	-	-	-	S	+	+
<u>Sau96I</u>	GGNCC	-	-	-	+	S	+
<u>TaqI</u>	*TCGA	+	S	S	+	+	+
<u>ThaI</u>	CGCG	-	-	S ²	-	S	+

Symbols used:

+, digestion with similar results when 1 μ g of DNA was digested under standard conditions for 1 h with 1.5 U of enzyme or for 4 h with 5 U of enzyme.

-, no digestion in the presence of five-fold excess of enzyme for 4 h.

S, slow digestion.

*, implies that when this residue is methylated, this sequence no longer serves as a recognition site for the indicated enzyme.

¹Digestion was incomplete even after 4 h of incubation as evidenced by most of the DNA being in a smear at the top of the gel and no discrete bands in the gel.

²Discrete fragment bands were obtained but only a portion of the DNA was digested even after incubation with a five-fold excess of enzyme for 4 h or overnight. Most or much of the DNA co-banded with untreated DNA.

Table 2. Digestion of highly modified DNA with restriction endonucleases with recognition sites containing five or six base pairs

Type of restriction endonuclease	Recognition sequence	DNA with modified C residues		DNA with modified T residues			Normal DNA (control) λ
		XP12	T4	SP15	SP01	PBS1	
<u>Ava</u> II	GG $\overset{A}{T}$ CC	-	-	-	S	+	
<u>Bam</u> HI	GGATCC	-	-	-	-	+	
<u>Bcl</u> I	TGATCA	-	-	-	S ¹	+	
<u>Bgl</u> I	GCCNNNNNGGC	-	-	-	S ²	S	+
<u>Bgl</u> II	AGATCT	-	-	-	S	-	
<u>Bst</u> EII	GGTNACC	S	-	-	S	S	+
<u>Bst</u> NI	CC $\overset{A}{T}$ GG	S	-	S ¹	+	S	+
<u>Eco</u> RII	CC $\overset{*}{A}$ $\overset{*}{T}$ GG	-	-	-	S ²	S	+
<u>Eco</u> RI	GA $\overset{*}{A}$ TC	-	-	-	S ²	S	+
<u>Hae</u> II	PuGCGCPy	-	-	-	S	S	+
<u>Hinc</u> II	GTPyPuAC	-	-	-	S	S	+
<u>Hind</u> III	$\overset{*}{A}$ AGCTT	-	-	-	S	S	+
<u>Hpa</u> I	CTTA $\overset{*}{A}$ C	-	-	-	S	-	+
<u>Sst</u> I	GAGCTC	-	-	-	-	S ¹	+
<u>Sst</u> II	CCGCGG	-	-	-	S ¹	S	+
<u>Xba</u> I	TCTAGA	-	-	-	S	S	
<u>Xor</u> II	CGATCG	-	-	-	-	-	+

Symbols used:

See Table 1.

and completeness of cleavage of λ DNA by most of these enzymes and obtained the expected results (Tables 1 & 2). As expected, only approximately half of the λ DNA preparation was extensively digested by MboI and EcoRII. This partial resistance is due to incomplete methylation of A residues in GATC sequences and the internal C residues in CC $\overset{A}{T}$ GG recognition sequences of MboI and EcoRII, respectively, in the E. coli host used for propagation of λ (1,22). Susceptible sites in these DNAs were cleaved at normal rates. In contrast, all of the examined highly modified DNAs were not detectably cleaved or were cleaved at an abnormally slow rate (Tables 1 and 2, Figs. 1 and 2) by many of the enzymes.

Figure 1. Digestion of SP01 and SP15 DNAs by several restriction endonucleases. Each set of four samples containing 1 μ g of DNA was digested with 1.5 unit for 1 h, 5 units for 1 h, 1.5 units for 2 h, or 5 units for 4 h, and electrophoresed in consecutive channels. Samples 1-4, SP01 DNA with CfoI; 5-8, SP01 DNA with HaeIII; 9-12, SP01 DNA with HpaII; 13-16, SP15 DNA with HpaII; 17-20, SP15 DNA with BstNI; and 21-23, SP01 DNA with BstNI.

Three of the highly modified DNAs were highly resistant to cleavage by most of the thirty enzymes tested (Tables 1 & 2). These DNAs were XP12 with 34 mole % m^5C (10), T4 with 17 mole % glucosylated hm^5C (11), and SP15 with 19 mole % phosphoglucuronated and glucosylated hp^5U (15). Phage T4 DNA was cleaved by only one of the examined enzymes; eight of these have been previously shown to be unable to cleave T4 DNA (24,25). With the exception of TaqI on XP12 DNA as a substrate, those few enzymes which cleaved these three phage genomes did so at an abnormally slow rate (Tables 1 & 2). In some cases in which the DNAs were cleaved, digestion was apparently incomplete even after incubation with an excess of enzyme. Incomplete digestion was evidenced by much undegraded or smeared DNA near the top of the agarose gel after electrophoresis (Fig. 1, channels 17-20; Tables 1 & 2). In other cases, these DNAs were extensively digested albeit slowly by the enzymes to

Figure 2. Digestion of XP12 and λ DNAs with several restriction endonucleases. Channels 1-4 (XP12 DNA) and 5-8 (λ DNA), digestion of 1 μ g of DNA with BstEII using 1.5 units for 1 h, 5 units for 1 h, 1.5 unit for 2 h, or 5 units for 2 h, channels 1-4 or 5-8, consecutively. Channel 9, digestion of 1 μ g of XP12 DNA plus 1 μ g of λ DNA with 1.5 unit of BstEII for 1 h. Channel 10 shows markers generated from digestion of λ with HindIII; the fragments have the following lengths: 23.7, 9.5, 6.6, 4.3, 2.1 and 1.9 bp. Channels 11 and 12 show a digest of 1 μ g of XP12 DNA incubated with 1.5 unit of BstNI for 1 h or 5 units for 4 h.

which they were susceptible (Fig. 2, channels 11 and 12; Tables 1 & 2). Because we did not know the density of restriction sites or if a small percentage of these sites were in an unusually unfavorable sequence environment (23), we scored digestion as slow, only if considerable differences were found between digestion of 1 μ g of DNA samples incubated with 1.5 U of enzyme for 1 h and those incubated with 5 U for 4 h.

Enzymes which digested XP12 DNA, although more slowly than normal, were BstNI, HinfI, BstEII, and MboI (Tables 1 & 2). The first two of these enzymes cleaved ϕ X174 RFI containing almost all the C residues in one strand replaced by m^5C residues (26). Both BstNI and MboI had not been tested previously on an m^5C -rich substrate although BstNI (and not EcoRII) was found

to cleave the following methylated form of its recognition sequence found in DNA from *E. coli* *mec*⁺ cells : Cm⁵C $\frac{A}{T}$ GG (27). That HincII and HpaI cleave hemimethylated ϕ X174 RFI but not XP12 DNA might be due to a lack of their recognition sequences in XP12 DNA or to differences in the ability of fully methylated DNA and hemi-methylated DNA to serve as substrates. MboI and TaqI, both of which cleaved XP12 DNA, are known to be inhibited by methylation of A residues in their recognition sequence (1). However, EcoRI, HindIII, and HpaI, which are similar inhibited by methylation of A residues (1) did not cleave XP12 DNA (Tables 1 & 2).

Digestion of PBS1 and SP01 DNAs with restriction endonucleases. PBS1 DNA with 36 mole % U (13) and SP01 DNA with 31 mole % hm⁵U (28) were cleaved by most of the examined restriction endonucleases but often more slowly than a normal DNA substrate was (Tables 1 & 2). A number of the enzymes tested did not detectably cleave these DNAs or cleaved them in an obviously incomplete manner especially in the case of enzymes with recognition sites that contained five or six base pairs (Tables 1 & 2). The need, in many cases, for increased incubation times or enzyme concentrations is consistent with previous studies in which SP01 and PBS1 DNAs were incubated with fifteen and seven of these enzymes respectively (25,29,30).

To assure that no extraneous inhibitors were present in the highly modified DNA preparations, mixing experiments were performed. The following DNAs (1 μ g) and enzymes (1.5 or 2 U) were incubated in the presence of 1 μ g of λ or pBR322 as an internal positive control: SP01 DNA and HaeII; PBS1 DNA and HpaI, Sau96I, ThaI, BglII, or EcoRI, as well as XP12 DNA and DdeI, HaeIII, Hinfl, Sau96I, ThaI, AvaII, BamHI, BstEII, BstNI, HaeII, SstI, HincII, or SstII; T4 DNA and HindIII or BglII; and SP15 DNA and BstEII. In all cases, the normal DNA appeared to be completely digested while the highly modified DNA was not detectably hydrolyzed or was digested to a much lower extent than was the normal DNA (e.g., Fig. 2, channels 1-9). These results also imply that in the cases tested, the presence of non-substrate DNAs did not detectably inhibit hydrolysis of substrate DNAs.

Phages PBS1 and SP15 are generalized transducing phages (31) and phage XP12 might also be one because ~2% of the DNA in purified phage preparations is cytosine-containing DNA apparently derived from the host chromosome (10). Since generalized transduction involves the packaging of randomly chosen host sequences and since the percentage of host DNA in our phage preparations is small, this host DNA did not make a significant contribution to the gel profiles. For example, we determined that by high performance liquid chrom-

atography analysis of DNA digests (11), <2% as much T (derived from host DNA) as U or as hm⁵U (derived from the phage genomes) was present in our PBS1 and SP01 DNA preparations, respectively. The incomplete digestion noted in Tables 1 & 2 refers to most of the DNA having been in a smear at the top of the gel or the conversion of a small amount of DNA to discrete bands.

The relative ability of different enzymes to digest the variously modified phage DNAs depended on the individual enzyme. Although many of the enzymes digested both PBS1 and SP01 DNA at normal rates or both at slow rates, several other enzymes digested either SP01 DNA or PBS1 DNA better (Tables 1 & 2). Some isoschizomers also varied in their relative acceptance of different unusually modified DNA substrates. As expected, when digestion appeared to proceed to completion, limit digests of a given DNA by isoschizomers produced similar digestion patterns.

TaqI. TaqI was the only enzyme able to cleave all of these modified DNAs. The TaqI limit digests consisted exclusively of fragments of <7 kilobase pairs (kb) and most of them were <3 kb (<1 kb for XP12 DNA). Therefore, extensive digestion was obtained even though all copies of T4, XP12, SP01, and PBS1's TaqI sites and ~85% of the copies of SP15's TaqI sites should contain a modified pyrimidine. It had previously been reported that TaqI can cleave the sequence Tm⁵CGA in mammalian DNA containing m⁵C residues located predominantly in CG sequences (7). That TaqI cleaved XP12 DNA normally (Table 1) indicates that even methylation of all the C residues in a DNA molecule does not inhibit digestion of that DNA by TaqI. Also, complete replacement of T residues with U or hm⁵U residues did not prevent TaqI from extensively digesting DNA with apparently normal kinetics (Table 1). TaqI's ability to digest unusually modified DNA had also been seen in its repeated cleavage of phage fd RFI DNA which was ~90% substituted in one strand with 4-thiothymine residues (32).

Digestion with pancreatic DNase and snake venom phosphodiesterase. We have also studied the effect of extensive DNA modification on hydrolysis by two nucleases with relatively little sequence specificity, namely pancreatic DNase (DNase I) and snake venom phosphodiesterase. The former is active on double-stranded DNA as well as on single-stranded DNA and the latter only on single-stranded DNA. Table 3 shows that while the replacement of hm⁵U with T (SP01 DNA) did not decrease these hydrolysis rates, substitution of C with m⁵C (XP12 DNA) or phosphoglucuronated and glucosylated hp⁵U instead of T (SP15 DNA) caused a three- to five-fold decrease. The hydrolysis of uracil-

Table 3. Effect of DNA modification on relative initial rates of hydrolysis by DNase I and snake venom phosphodiesterase

DNA	DNase I		Venom phosphodiesterase	
	Rate ¹	(N) ²	Rate ³	(N)
Non-modified ⁴	1 ± 0.20	(8)	1 ± 0.39	(6)
Modified				
XP12	0.38 ± 0.07	(4)	0.21 ± 0.06	(2)
SP15	0.18 ± 0.08	(3)	0.28 ± 0.05	(2)
PBS1	0.07 ± 0.03	(2)		
SP01	1.42 ± 0.51	(2)	0.97	(1)
T4	0.81	(1)	0.07	(1)

¹Rates were measured by increase in absorbance at 260nm with time after enzyme addition. Only the initial, linear portion of the curve was used to calculate the rates.

²N = number of determinations.

³Hydrolysis was on heat-denatured DNAs.

⁴Non-modified DNAs were from *B. subtilis*, *X. oryzae*, salmon sperm, calf thymus, and T7 bacteriophage. These DNAs were chosen to provide a variety of GC contents.

rich PBS1 DNA with DNase I was much slower than that of normal DNAs.

Compared to normal DNAs, T4 DNA with glucosylated hm⁵C was hydrolyzed much more slowly by venom phosphodiesterase but not by DNase I.

DISCUSSION

Highly modified phage DNAs were shown to vary considerably in their digestion by different nucleases and T4, SP15, and XP12 DNAs were the most resistant. Since specific methylation of a C residue at the recognition site was already known to inhibit cleavage by *HpaII*, *MspI*, *HhaI*, *HaeIII*, *EcoRII*, and *Sau3A* (1,4,7,33), the lack of double-strand cleavage of XP12 DNA by these enzymes was expected. Nonetheless, we cannot exclude the possibility that XP12 DNA does not contain the recognition sequence for one or several of the twenty five enzymes which failed to cleave it. However, given its molecular weight of 3×10^7 and its base composition of 32% A + T (9), XP12 DNA would be predicted to have on the average about 150-600 copies of a four base pair recognition sequence and 4-70 copies of a six base pair recognition sequence, depending on the bases in the sequence. It is, therefore, extremely improbable that XP12 DNA lacks the recognition sites for more than a few of these enzymes. Since all of the other highly modified

DNAs tested have much larger molecular weights ($>10^8$; 16), a similar argument pertains to their resistance to hydrolysis. Furthermore, cytosine-containing T4 DNA obtained from a multiply mutant phage was shown to be cleaved by twelve of the enzymes listed in Table 2 (34-36).

Limited modification of the phage DNA by a sequence-specific methylase, (1,37) might explain resistance of a small number of these DNAs to digestion by a few of these enzymes. However, no more than a few DNA methylases of different specificity are found in a given infected (38) or uninfected bacterial cell. Therefore, in most cases, lack of cleavage of these DNAs must be due to their extensive modification.

The inhibitory modification of T4 and SP15 DNA could be either the presence of hm^5C and hp^5U , respectively, or the glucose (11) or glucose plus phosphoglucuronolactone attached to the base (15). Non-glucosylated hm^5C -containing T4 DNA from mutant phage as well as normally glucosylated T4 DNA is not a substrate for EcoRII, HindII and HindIII but is for EcoRI (24). It is, therefore, possible that either the sugar or sugar phosphate attached to the unusual base in T4 and SP15 DNAs or the hm^5C or hp^5U moieties themselves are responsible for the resistance of these DNAs to most of the tested restriction enzymes.

Slower than normal digestion or the lack of detectable cleavage of the highly modified DNAs could have been due to modification of residues within or outside of the recognition sequence. Nine of the enzymes contain recognition sites with only G-C base pairs. All but one of these enzymes were unable to hydrolyze SP15 DNA, whose only unusually modified residues is a derivative of T (Tables 1 & 2). Furthermore, under standard conditions, cleavage of SP01 and PBS1 by three and five of these enzymes, respectively, was at least partially inhibited even though these DNAs too have modified T rather than C residues (Tables 1 & 2). These are, therefore, additional examples of the previously detected influence of residues outside of the recognition sequence on the activity of various restriction endonucleases (3,23,32).

There is no correlation between the melting temperatures (16) of the DNAs analyzed and their susceptibility to digestion by restriction endonucleases. For example, XP12 DNA with the highest known melting temperature of any naturally occurring DNA (9) and SP15 DNA with the lowest melting temperature (39) were both highly resistant to cleavage by most of these enzymes. Also, no simple correlation of temperature used for digestion and digestibility was found. Although three of the five restriction enzymes

which cleaved XP12 DNA and three of the four which cleaved SP15 DNA were derived from extreme thermophiles and therefore used at 55°-65°, several enzymes derived from thermophiles did not cleave one or the other of these DNAs. Furthermore, extensive digestion of all the highly modified DNAs was obtained with TaqI at 37°C as well as at 65°C, although at a slower rate at the lower temperature as expected.

Modified bases at or adjacent to the recognition sites of many of these enzymes could interfere with enzyme binding or catalysis by inducing changes in conformation of the DNA, or the formation of improper protein-DNA contacts at the recognition and catalysis site. Also, modified bases outside of the recognition site could inhibit nonspecific binding of restriction enzymes, which might be preliminary to specific binding (40). Steric hindrance might (partially) explain the inhibition of digestion of XP12, SP01, and especially T4 and SP15 DNAs by some of the restriction enzymes. It might also explain the lower rate of digestion of some of these DNAs by snake venom phosphodiesterase and DNase I, which exhibit relatively little base specificity. It is also possible that the extensive modification of these phage DNAs alters their conformation. In preliminary studies of X-ray diffraction patterns of DNA fibers, XP12 DNA and especially SP15 DNA displayed peculiar features (Chandrasekaran, Arnott, Gama-Sosa, Ehrlich unpublished observations). Although XP12 DNA did give the B form in the presence of 1 M sodium salt, the occurrence of the A form under these conditions was more frequent; this contrasts with normal DNA, which gave predominantly the B form. Furthermore, only the B pattern instead of the expected A pattern was obtained from SP15 DNA in low salt concentration. There might also be subtle local conformational differences (41,42) in highly modified DNAs which affect hydrolysis by sequence-specific enzymes. Moreover, these enzymes distinguish the major bases from one another and at certain positions discriminate between C and m⁵C or A and m⁶A. Therefore, the modified bases at the recognition sequences of these unusual phage DNAs might simply not make proper contact with the enzymes' binding or catalysis sites. This is especially likely to be the case for at least some of these enzymes because, in the B conformation, the 5-position of C and T residues is prominently exposed (43,44). Hence, the substituents at the 5-position of these modified DNAs may preclude or retard normal interaction with the recognition surfaces of many of the restriction endonucleases.

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REFERENCES

1. Roberts, R. J. (1981) *Nucleic Acids Res.* 9, r75-r96.
2. Smith, H. O. (1979) *Science* 205, 455-462.
3. Modrich, P. (1979) *Quart. Rev. Biophys.* 12, 315-369.
4. Mann, M. B. and Smith, H. O. (1977) *Nucleic Acids Res.* 4, 4211-4221.
5. Jeitsch, S., Gunthert, U., and Trautner, T. A. (1981) *Nucleic Acids Res.* 9, 2753-2759.
6. Waalwijk, C. and Flavell, R. A. (1978) *Nucleic Acids Res.* 5, 3232-3236.
7. Streek, R. E. (1980) *Gene* 12, 267-275.
8. Kuo, T.-T., Huang, T.-C., and Teng, M.-H. (1968) *J. Mol. Biol.* 34, 373-375.
9. Ehrlich, M., Ehrlich, K., and Mayo, J. A. (1975) *Biochim. Biophys. Acta* 395, 109-119.
10. Kuo, K. C., McCune, R. A., Gehrke, C. W., Midgett, R., and Ehrlich, M. (1980) *Nucleic Acids Res.* 8, 4763-4776.
11. Lehman, I. R. and Pratt, E. A. (1960) *J. Biol. Chem.* 235, 3254-3259.
12. Kallen, R. G., Simon, M., and Marmur, J. (1962) *J. Mol. Biol.* 5, 248-250.
13. Takahashi, I. and Marmur, J. (1963) *Nature* 197, 794-795.
14. Hayashi, H., Nakanishi, K., Brandon, C., and Marmur, J. (1973) *J. Amer. Chem. Soc.* 95, 8749-8757.
15. Ehrlich, M. and Ehrlich, K. (1981) *J. Biol. Chem.* 256, 9966-9972.
16. Warren, R.A.J. (1980) *Ann. Rev. Microbiol.* 34, 137-158.
17. Boliver, F., Rodriquez, R. L., Greene, P. J., Betlech, M. C., Heynecker, H. L., Boyer, H. L., Crosa, J. H., and Falkow, S. (1977) *Gene* 2, 95-113.
18. Crosa, J. H., Luttrupp, L. K., and Falkow, S. (1975) *Proc. Natl. Acad. Sci. USA* 72, 654-658.
19. Holmes, D. S. and Quigley, M. (1981) *Anal. Biochem.* 114, 193-197.
20. Clewell, D. B. and Helinski, D. R. (1969) *Proc. Nat. Acad. Sci. USA* 63, 1159-1166.
21. Marmur, J. (1961) *J. Mol. Biol.* 3, 208-218.
22. Hughes, S. G. (1975) *J. Mol. Biol.* 98, 645-647.
23. Wang, R. Y.-H., Shedlarski, J. G., Farber, M. B., Kuebbing, D., and Ehrlich, M. (1980) *Biochim. Biophys. Acta* 606, 371-385.
24. Kaplan, D. A. and Nierlich, D. P. (1975) *J. Biol. Chem.* 250, 2395-2397.
25. Berkner, K. L. and Folk, W. R. (1979) *J. Biol. Chem.* 254, 2551-2560.
26. Gruenbaum, Y., Cedar, H., and Razin, A. (1981) *Nucleic Acids Res.* 9, 2509-2513.
27. Hattman, S., Gribbin, C., and Hutchinson, C. A. (1979) *J. Virol.* 32, 845-851.
28. Truffant, N., Revet, B., and Soulie, M. (1970) *Eur. J. Biochem.* 15, 391-400.
29. Pero, J., Hannett, N. M., and Talkington, C. (1979) *J. Virol.* 31, 156-171.
30. Cregg, J. M. and Stewart, C. R. (1978) *Virology* 85, 601-605.
31. Hemphill, H. E. and Whitely, H. R. (1975) *Bacteriol. Rev.* 39, 257-315.
32. Hofer, B. and Koster, H. (1981) *Nucleic Acids Res.* 9, 753-761.
33. Ehrlich, M. and Wang, R. Y.-H. (1981) *Science* 212, 1350-1357.

34. Marsh, R. C. and Hepburn, M. L. (1981) *J. Virol.* 38, 104-114.
35. Hanggi, U. J. and Zachau, H. G. (1980) *Gene* 9, 271-285.
36. Pribnow, D., Sigurdson, D. C., Gold, L., Singer, B. S., Napoli, C., Broosius, J., Dull, T. J., and Noller, H. F. (1981) *J. Mol. Biol.* 149, 332-376.
37. Gunthert, R., Freund, M., and Trautner, T. A. (1981) *J. Biol. Chem.* 256, 9340-9345.
38. Hattman, S., van Ormondt, H., and de Waard, A. (1978) *J. Mol. Biol.* 119, 361-376.
39. Tyeryar, F. J., Taylor, M. J., Lawton, W. D., and Goldberg, I. D. (1969) *J. Bacteriol.* 100, 1027-1036.
40. Woodhead, J. L. and Malcolm, A. D. B. (1980) *Nucleic Acids Res.* 8, 389-402.
41. Leslie, A. G. W., Arnott, S., Chandrasekaran, R., and Ratliff, R. L. (1980) *J. Mol. Biol.* 143, 49-72.
42. Lomonosoff, G. P., Butler, P. J. G., and Klug, A. (1981) *J. Mol. Biol.* 149, 745-760.
43. Alden, C. J. and Kim, S.-H. (1979) *J. Mol. Biol.* 132, 411-434.
44. Seeman, N. C., Rosenberg, J. M., and Rich, A. (1976) *Proc. Natl. Acad. Sci. USA* 73, 804-808.