Proc. Natl. Acad. Sci. USA
Vol. 80, pp. 7400–7404, December 1983
Biochemistry

Purification and characterization of the unusual deoxynucleoside, α-*N*-(9-β-D-2′-deoxyribofuranosylpurin-6-yl)glycinamide, specified by the phage Mu modification function

(DNA modification/HPLC/mass spectrometry)

DAVID SWINTON*, STANLEY HATTMAN*, PAMELA F. CRAIN†, CHIN-SHU CHENG†, DAVID L. SMITH†, AND JAMES A. MCCLOSKEY†

*Department of Biology, University of Rochester, Rochester, NY 14627; and †Departments of Biochemistry and Medicinal Chemistry, University of Utah, Salt Lake City, UT 84112

Communicated by S. E. Luria, August 25, 1983

ABSTRACT **Bacteriophage Mu encodes a protein that modifies ≈15% of DNA adenine residues to a new and unusual form. Modified DNA was enzymatically digested to deoxynucleosides, and the products were fractionated by HPLC. A modified adenine nucleoside, designated dA'_x, was purified and its molecular structure was established by mass spectrometry. We show that dA'_x is α-*N*-(9-β-D-2′-deoxyribofuranosylpurin-6-yl)-glycinamide. The dA'_x obtained from DNA was indistinguishable from the synthetic product with respect to its chromatographic behavior (HPLC and gas chromatography) and mass spectrum. Acid hydrolysis degrades dA'_x to produce N^6-carboxymethyladenine; this compound corresponds to the base A_x observed in earlier studies.**

Bacteriophage Mu is known to control a DNA modification function (1) that protects its DNA against restriction by certain site-specific nucleases (2, 3). The modification usually requires expression of both phage and host genes: (*i*) the phage Mu *mom*$^+$ gene (1, 4), which is located at the rightmost end of the genetic map; (*ii*) at least one other phage Mu (*trans*-acting) gene (5); and (*iii*) the host *Escherichia coli dam*$^+$ gene (6, 7), which specifies a DNA-adenine methylase (8) that modifies the sequence G-A-T-C to G-m^{6}A-T-C (9, 10). It has recently been shown that the *dam*$^+$ methylase exerts a positive regulatory role in transcription of the phage Mu *mom* gene (11). Previously, the DNA modification was partially characterized, showing that ≈15% of the adenine residues are modified (12) and that these residues are in specific sequences (3, 13). The modified base (designated A_x), observed in mild acid hydrolysates, was shown to contain a free carboxyl residue (12). The present communication reports the purification and molecular characterization of the modified deoxynucleoside. Mass spectrometric analysis identified the structure as α-*N*-(9-β-D-2′-deoxyribofuranosylpurin-6-yl)glycinamide. Further investigation revealed that acid hydrolysis degrades this compound to produce N^6-carboxymethyladenine; this base corresponds to the A_x observed in earlier studies (12).

MATERIALS AND METHODS

Media and Chemicals. LB broth contains (per liter) 10 g of Bacto-tryptone (DIFCO), 5 g of yeast extract (DIFCO), 5 g of NaCl, adjusted to pH 7.0 with NaOH; 1× NaCl/Cit is 0.15 M NaCl/0.015 M Na citrate, adjusted to pH 7.0; TEN buffer is 10 mM Tris·HCl/10 mM Na_2EDTA/0.15 M NaCl, pH 8.0.

Bacterial Strains. *E. coli* strains QD 5003 *sup* (Mu*cts*62 *lys*1025) and ND40 (*trp*$^-_{am}$/F′ 113 *sup*E) were kindly provided by A. Bukhari. Phage Mu (*cts*62 *lys*1025) was obtained by thermal induction of QD5003 and used to lysogenize ND40. One lysogen was isolated that did not grow in the absence of exogenous tryptophan, presumably because of loss of the F′ *sup*E factor. This strain did not lyse after thermal induction of Mu prophage, an observation consistent with the failure to suppress the amber mutation in the Mu *lys* gene. Separate experiments showed that the host DNA was subject to Mu modification after prophage induction (unpublished data).

Preparation of Modified Host DNA. Ten 500-ml LB broth cultures of ND40 *trp*$^-_{am}$ (Mu*cts*62 *lys*1025) were grown in 2-liter flasks at 32°C. At a titer of ≈3 × 10^8 cells per ml, the flasks were transferred to 42°C; after 30 min of incubation with shaking they were transferred to 37°C for 120 min. The cells were harvested by centrifugation and washed once in 1× NaCl/Cit, and the pellets were stored frozen at −20°C; the yield was ≈15 g (wet weight) of cells.

The pellet was thawed and suspended in 150 ml of TEN buffer; 50 mg of crystalline egg white lysozyme was added, and the culture was gently swirled at 37°C. Lysis was completed by addition of 8 ml of 10% (wt/vol) Sarkosyl NL 97. Pronase (final concentration, 300 μg/ml) was added and incubation continued for 3 hr at 37°C. Deproteinization was continued by extractions with phenol and then chloroform/isoamyl alcohol, 24:1 (vol/vol). The aqueous phase was covered with 2 vol of 95% ethanol and the DNA was "spooled" on glass rods as described by Marmur (14). After dissolution in 50 ml of 0.1× TEN buffer, residual RNA was enzymatically digested as described above, and this was followed by another Pronase digestion. The sample was diluted and extracted with chloroform/isoamyl alcohol and precipitated with ethanol as described above. After suspension in 50 ml of 0.1× NaCl/Cit, 20 ml was taken for a third extraction, precipitation, and dissolution as described above, and then dialyzed against H_2O. Through all steps, the DNA solution was extremely viscous even after prolonged dissolution by vigorous stirring with a magnetic spin bar. The 5-liter culture yielded ≈40 mg of DNA at A_{260}/A_{280} = 1.80 and A_{260}/A_{230} = 2.0.

Purification of dA'_x. Modified *E. coli* DNA (2–4 mg) was dissolved in 1 ml of 50 mM Tris·HCl (pH 8.5). The solution was made 7 mM in $MgCl_2$, 5 mM in $CaCl_2$, and 50 units of DNase I (Worthington) was added. After 4 hr of incubation at 50°C, 2.5 units of snake venom phosphodiesterase (Worthington) was added, and the incubation continued overnight. An additional 2.5 units of snake venom phosphodiesterase was added along with 0.1 unit of bacterial alkaline phosphatase (Worthington), and the mixture was incubated another 4 hr. Three volumes of 95% ethanol was added and the mixture was chilled to −20°C for 1 hr. After centrifugation, the nucleoside-containing supernatant was collected and lyophilized to a volume of 80 μl or

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

less. The nucleosides were separated by the HPLC method of Singhal (15), modified by using a 0.4 × 100 cm column of Aminex A-7 and a flow rate of 12 ml/hr. This procedure yields the modified nucleoside, dA'_x, free of other deoxyribonucleosides but does not separate it from adenosine, which may be present as a contaminant. Fractions containing the modified deoxyribonucleoside were concentrated by lyophilization, dissolved in 50 mM KH_2PO_4/5% methanol, pH 4.2, and chromatographed isocratically in the same buffer at 15 ml/hr by the method of Kuo *et al.* (16). This chromatography completely separates dA'_x and adenosine but suffers from a relatively low capacity.

Synthesis of α-*N*-(9-β-D-2′-deoxyribofuranosylpurin-6-yl)-glycinamide. 6-Chloro-9-(β-D-2′-deoxyribofuranosyl)purine (17) (90 mg; 0.33 mmol) was dissolved in 1 ml of dry dimethylformamide. After addition of 1 ml of aqueous glycinamide·HCl (Sigma) (73.7 mg; 0.66 mmol) and sodium bicarbonate (84 mg; 1 mmol), the suspension was heated in a 95°C oil bath for 5 hr with constant stirring. Solvent was removed under reduced pressure and the residue was treated with ≈5 ml of methanol. The resulting slurry was filtered; concentration of the filtrate yielded 71 mg of product, which was washed with cold H_2O and then recrystallized from water. Total yield of material was 70%; there was no attempt to maximize the yield. The product was characterized as follows: mp 210–211°C (dec); ^1H NMR (^2H_2O): δ 2.56 (m, 1, $H_{2'}$), 2.83 (m, 1, $H_{2''}$), 3.81 (m, 2, $H_{5'}$, and $H_{5''}$), 4.20 (d, 1, $H_{4'}$), 4.29 (m, 3, $H_{3'}$, and $=NCH_2CO-$), 6.45 (t, 1, $H_{1'}$), 8.23 (s, 1, H-8, partially exchangeable), 8.29 (s, 1, H-2); M_r of the trimethylsilyl derivative by peak matching, 596.2804; 596.2812 is required for $C_{24}H_{48}N_6O_4Si_4$.

Instrumentation. All mass spectra were recorded with a Varian MAT 731 instrument at 70 eV ionizing energy, 8 kV accelerating voltage, and 270°C ion source temperature. Sample introduction was by probe inlet; aliquots of derivatization mixtures were placed in sample crucibles, and reagents and solvents were removed in the probe vacuum lock before sample introduction. Mass spectra were recorded in the temperature range 70–150°C. Low-resolution spectra were acquired by using a Varian SS100C data system. High-resolution data were acquired by peak matching in selected cases or by photographic recording of the full spectrum using evaporated AgBr photoplates (Ionomet, Brighton, MA) at an instrumental resolving power of 15,000. Photographic data were analyzed using a Gaertner M1205PC comparator interfaced to a Varian SS100C data system.

Proton magnetic resonance spectra were taken on a JEOL FX-270 instrument at 270 MHz and 23°C probe temperature. Chemical shifts are reported relative to sodium 2,2-dimethyl-2-silapentane-5-sulfonate (Stohler Isotope Chemicals, Waltham, MA).

Preparation of Trimethylsilyl Derivatives for Mass Spectrometry. Aliquots of a water solution of dA'_x equivalent to 2–3 μg were placed in melting point capillary tubes and carefully dried. Nine microliters of *N,O*-bis(trimethylsilyl)trifluoroacetamide containing 1% trimethylchlorosilane (Sylon BFT; Supelco, Bellefonte, PA) and 1 μl of dry pyridine (or dimethylformamide) were added to the tube, which was sealed and heated for 1 hr at 85°C.

Unfractionated 5′-deoxyribonucleotides from Mu DNA digests were trimethylsilylated as described for derivatization of ribonucleotides (18). The equivalent of 10 μg of digest was transferred to a 4-mm i.d. tube prepared from Pyrex tubing, and treated with 10 μl of Sylon BFT and pyridine (1:1) for 30 min at 85°C.

Preparation of 5′-Deoxyribonucleotides from Mu DNA. Mu DNA was digested to 5′-deoxyribonucleotides by treatment of 50 μg of DNA (0.7 mg/ml in 20 mM ammonium bicarbonate, pH 7.7) with 2 μg of DNase I (Worthington) (1 mg/ml in 20 mM ammonium formate, pH 7.2) and allowing digestion to proceed overnight at 37°C. Four microliters of 0.2 M triethylammonium bicarbonate (pH 8.0) was added to the solution, along with 2 μg of snake venom phosphodiesterase (1 mg/ml in 0.2 M ammonium carbonate); digestion was continued for 6 hr at 37°C.

RESULTS

Purification and Characterization of dA'_x. In an earlier study on phage Mu DNA modification, an unusual base, designated A_x, was observed in mild acid hydrolysates of modified DNA; this base was shown to contain a free carboxyl residue (12). After enzymatic digestion of modified DNA, HPLC analysis revealed the presence of a new deoxynucleoside species, dA'_x (Fig. 1). The chromatographic data were not consistent with dA'_x containing a free carboxyl group, so it was unlikely that dA'_x was simply the deoxynucleoside of A_x. However, mild acid hydrolysis of dA'_x led to the appearance of A_x. Thus, it became evident that A_x is a degradation product of the acid-labile parent compound, dA'_x. Therefore, it was of interest to purify and characterize dA'_x.

The modified deoxynucleoside was purified by HPLC and the trimethylsilyl derivatives were made for mass spectrometric analysis (see *Materials and Methods*). The low-resolution mass spectrum of the trimethylsilyl derivative of dA'_x is shown in Fig. 2. The molecular ion (M) of the major product is indicated at m/z 596, accompanied by an ion 15 mass units lower (m/z 581), resulting from characteristic loss of a methyl radical from a trimethylsilyl function (18). Trace amounts of an ion 72 mass units higher than M (at m/z 668) were occasionally observed, indicating incorporation of an additional trimethylsilyl group. Ions of m/z 170, 155, and 103 are indicative of an unmodified sugar function (18); the deoxyribose fragment, m/z 261, is not abundant. The difference in mass between the molecular ion and the sugar fragment represents the base fragment (335 mass units), which characteristically (19) appears with a rearranged hydrogen, at m/z 336.

Compositions of these and other ions in the spectrum were obtained from exact mass values measured by high-resolution mass spectrometry. The computer-derived compositions for the measured mass of the molecular ion (596.2815) were screened for plausible compositions using the approach of Crain *et al.*

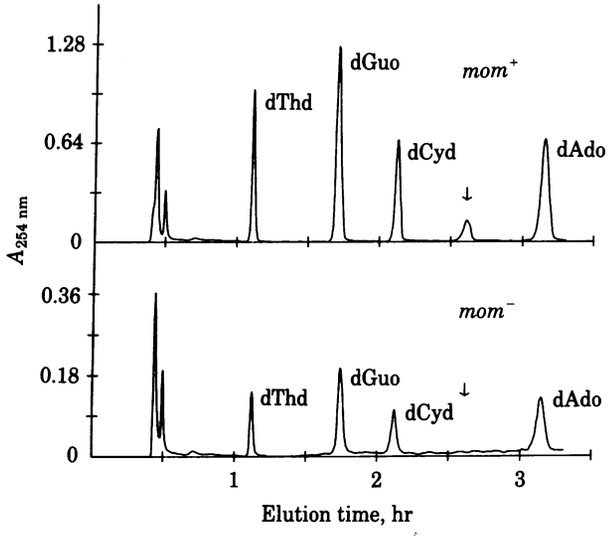

FIG. 1. HPLC profiles of enzymatic digests of modified Mu mom^+ and unmodified Mu mom^- DNAs. Arrow denotes the elution position of dA'_x.

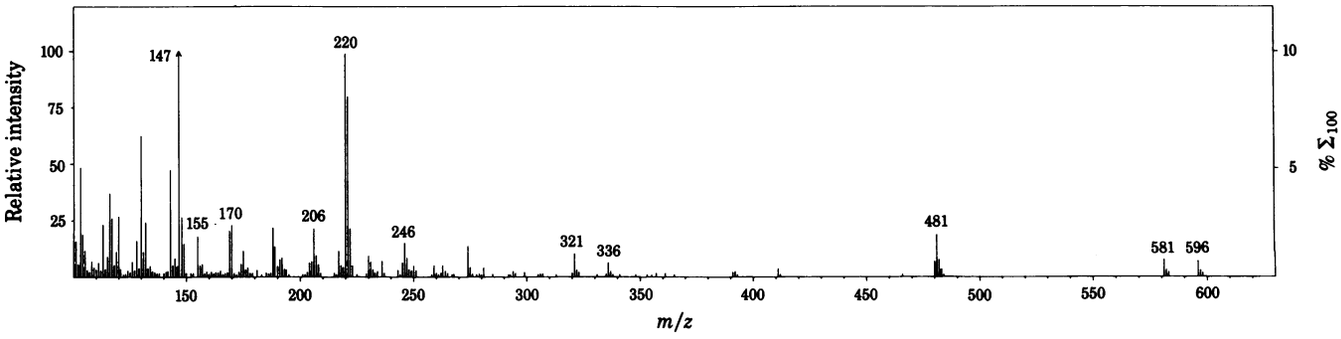

FIG. 2. Mass spectrum (70 eV) of the trimethylsilyl derivative of a modified deoxynucleoside isolated from bacteriophage Mu DNA.

(20). Four compositions were found to be consistent with a substituted deoxyadenosine residue. Two were eliminated as chemically implausible, and a third required the presence of sulfur, which is not evident in the low-resolution mass spectrum by the required isotopic pattern. The remaining composition corresponded to a tris(trimethylsilyl) derivative of deoxyadenosine and the elements $C_2H_2NOSi(CH_3)_3$. Compositions of major fragment ions in the low-resolution mass spectrum that are consistent with the probable molecular compositions are given in Table 1.

The arrangement of atoms in the added substituent(s) and their disposition were deduced as follows.

(*i*) Substitution at positions 2 or 8 in the adenine ring is ruled out from earlier work (12) showing retention of label in A_x base derived from acid digests of Mu DNA containing biologically incorporated [2-^{3}H]adenine or [8-^{3}H]adenine.

(*ii*) The major fragment of *m/z* 480 (accompanied by a rearranged H yielding *m/z* 481) is equivalent to loss of a 116-mass-unit fragment from M_x, the exact mass of which supports the composition $CONHSi(CH_3)_3$. The elemental compositions of these ions are consistent with ions A and A′ (Fig. 3, structure **1**) (here shown as a N-6 substituent, although substitution at N-1 or N-3 is not ruled out by mass spectral data alone). An analogous ion, *m/z* 220, results from cleavage of the glycosidic bond and loss of the 116 mass-unit fragment.

(*iii*) Although structure **2** yields the same ion, it is eliminated because the number of blocking groups (trimethylsilyl) incorporated should be one fewer than the four observed, because of the low probability of reaction of the designated secondary nitrogen during derivatization.

(*iv*) Placement of substituents as in structure **3** would yield an ion of *m/z* 494, which is not observed. The mass spectrum of the trimethylsilyl derivative of a related compound, *N*-methyl-*N*-[(9-β-D-ribofuranosylpurin-6-yl)carbamoyl]threonine, mt^{6}A, (21) does not undergo the illustrated cleavage.

Table 1. Diagnostic ions in the high-resolution mass spectrum of the trimethylsilyl derivative of modified deoxynucleoside dA'_x

Ion	Composition	Observed mass	Error
M	$C_{24}H_{48}N_6O_4Si_4$	596.2815	−0.3
M − CH_3	$C_{23}H_{45}N_6O_4Si_4$	581.2583	+0.5
A + H	$C_{20}H_{39}N_5O_3Si_3$	481.2367	+0.7
A	$C_{20}H_{38}N_5O_3Si_3$	480.2269	−1.2
B + H	$C_{13}H_{24}N_6OSi_2$	336.1582	+3.2
B + H − CH_3	$C_{12}H_{21}N_6OSi_2$	321.1334	+1.9
B + H − $(CH_3)_3SiOH$	$C_{10}H_{14}N_6Si$	246.1057	+0.9
A′	$C_9H_{14}N_5Si$	220.1005	−1.2
Sugar − H − $(CH_3)_3SiOH$	$C_8H_{14}O_2Si$	170.0764	+0.1
Sugar − H − $(CH_3)_3SiOH$ − CH_3	$C_7H_{11}O_2Si$	155.0527	−0.1
m/z 116	$C_4H_{10}NOSi$	116.0538	+0.6

B refers to the base moiety of structure 1. Error (in mass milliunits) determined by subtracting calculated mass from observed mass.

To preclude the possibility that dA'_x is an artifact of degradation from sample handling during isolation (pH 10), an unfractionated enzymatic digest of Mu mononucleotides was directly examined by mass spectrometry after silylation. The required peaks for M (*m/z* 748) and M-CH_3 (*m/z* 733) ions, and for the diagnostic fragment ion *m/z* 633 (analogous to *m/z* 481 in Fig. 2), were observed as shown in Fig. 5. These ions do not correspond to any ions from the four major deoxynucleosides (18) or potential nucleotide contaminants from RNA.

DISCUSSION

Bacteriophage Mu controls an unusual DNA modification function that is active on both host and viral genomes. The product of the modification reaction has been purified by HPLC and its molecular structure elucidated by mass spectrometry. The modified deoxynucleoside dA'_x was shown to be α-*N*-(9-β-D-2′-deoxyribofuranosylpurin-6-yl)glycinamide. This is the first known

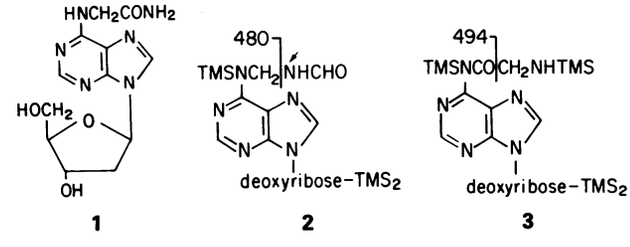

FIG. 3. Possible structures of major fragment ions in the mass spectrum of the trimethylsilyl (TMS) derivative of dA'_x (from Fig. 2).

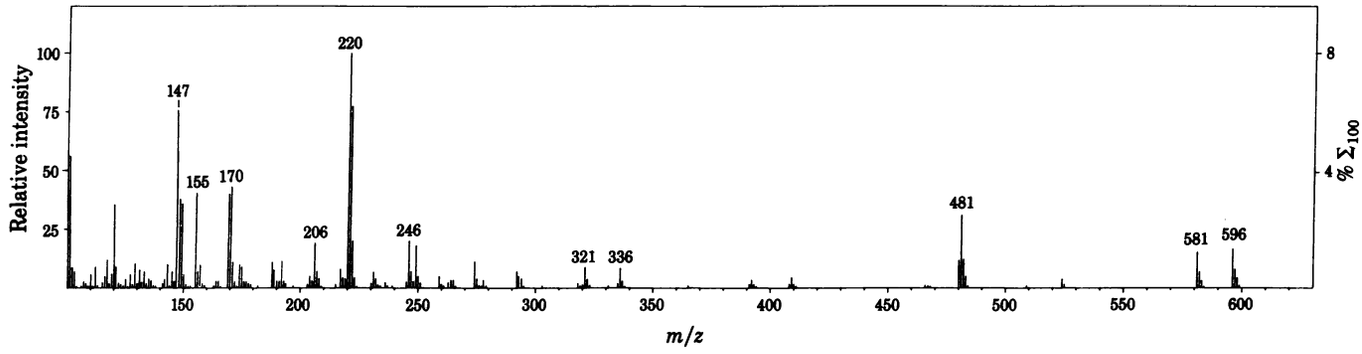

FIG. 4. Mass spectrum (70 eV) of the trimethylsilyl derivative of synthetic α-*N*-(9-β-D-2′-deoxyribofuranosylpurin-6-yl)glycinamide.

example of a hypermodified purine in DNA. There have been reports of other modified purines in DNA (23, 24) in addition to the well known case of N^6-methyladenine. For example, it was claimed (24) that 1-methyladenine is present in salmon sperm DNA, based on a method of analysis in which bases from DNA are liberated by pyrolysis (25). A later study (26) described the presence of a similar amount of m^1Ade in poly(dA-dT) using the same method of analysis, from which it was concluded that a signal for m^1Ade in DNA pyrolysates does not require its presence in the DNA and implies that it is formed thermally. In the absence of any other corroborating evidence for m^1Ade in DNA, it cannot, therefore, be assumed to be a naturally occurring DNA constituent. There are reports describing the presence of 7-methylguanine in phage DDVI (27) and 2-aminoadenine in cyanophage S-2L (28). In the former case, 7-methylguanine was present as a minor constituent; in contrast, 2-aminoadenine completely replaces adenine in phage S-2L. It would be of interest to see whether these findings can be corroborated by others.

In an earlier study of the Mu modification, an unusual base containing a free carboxyl group was observed after acid depurination of Mu DNA (12). In experiments not reported in this communication, we showed that this base, designated A_x, is N^6-carboxymethyladenine; this corresponds to the deaminated form of A'_x. In A'_x the acetamido group on the N-6 of adenine is accommodated in the major groove of the B-DNA helix. However, by analogy to N^6-methyladenine (29, 30), we would expect this group to destabilize the helix. In fact, the melting temperature (T_m) of modified Mu DNA is almost 5°C lower than the T_m for unmodified Mu DNA (31). It is remarkable that Mu DNA contains 15% of its adenine residues hypermodified to A'_x and, yet, the phage remains viable. In contrast, preliminary studies suggest that modification of the host DNA may be lethal (31); perhaps that is why phage Mu has evolved elaborate regulatory controls over the expression of this function (3, 31, 32). It seems reasonable to expect that with such a large fraction of modified bases, some DNA–protein interaction(s) critical for expression of an essential host gene will be impaired. Currently, we have no clue as to the donor substrate in the modification reaction. However, recent studies indicate that it is derived from normal cellular metabolite(s) and not synthesized under control of a phage gene (unpublished observation).

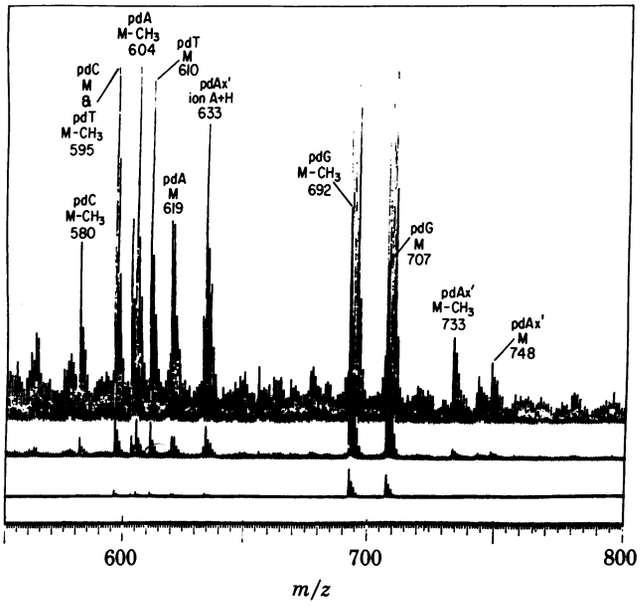

FIG. 5. Oscillographic recording of the molecular ion region of deoxynucleotides from a trimethylsilylated enzymatic digest of bacteriophage Mu DNA. The spectrum was recorded at a direct probe temperature of 140°C.

This work was supported by Public Health Service Grant GM-26892.

1. Toussaint, A. (1976) *Virology* **70**, 17–27.
2. Allet, B. & Bukhari, A. I. (1975) *J. Mol. Biol.* **95**, 529–540.
3. Kahmann, R. (1982) *Cold Spring Harbor Symp. Quant. Biol.* **47**, 639–646.
4. Toussaint, A., Desmet, L. & Faelen, M. (1980) *Mol. Gen. Genet.* **177**, 351–353.
5. Chaconas, G., de Bruijn, F. J., Casadaban, M., Lupski, J. R., Kwoh, T. J., Harshey, R. M., DuBow, M. S. & Bukhari, A. I. (1981) *Gene* **13**, 37–46.
6. Toussaint, A. (1977) *J. Virol.* **23**, 825–826.
7. Khatoon, H. & Bukhari, A. I. (1978) *J. Bacteriol.* **136**, 423–428.
8. Marinus, M. G. & Morris, N. R. (1973) *J. Bacteriol.* **114**, 1143–1150.
9. Lacks, S. & Greenberg, B. (1977) *J. Mol. Biol.* **114**, 153–168.
10. Hattman, S., Brooks, J. E. & Masurekar, M. (1978) *J. Mol. Biol.* **126**, 367–380.
11. Hattman, S. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 5518–5521.
12. Hattman, S. (1979) *J. Virol.* **32**, 468–475.
13. Hattman, S. (1980) *J. Virol.* **34**, 277–279.
14. Marmur, J. (1961) *J. Mol. Biol.* **3**, 208–218.
15. Singhal, R. P. (1972) *Arch. Biochem. Biophys.* **152**, 800–810.
16. Kuo, K. C., McCune, R. A., Gehrke, C. W., Midgett, R. & Ehrlich, M. (1980) *Nucleic Acids Res.* **8**, 4763–4776.
17. Robins, R. & Basom, G. L. (1978) in *Nucleic Acid Chemistry: Improved and New Synthetic Procedures, Methods and Techniques*, eds. Townsend, L. B. & Tipson, R. S. (Wiley Interscience, New York), Part 2, pp. 601–606.
18. Lawson, A. M., Stillwell, R. N., Tacker, M. M., Tsuboyama, K. & McCloskey, J. A. (1971) *J. Am. Chem. Soc.* **93**, 1014–1023.
19. McCloskey, J. A. (1974) in *Basic Principles in Nucleic Acid Chemistry*, ed. Ts'o, P. O. P. (Academic, New York), Vol. 1, pp. 209–309.
20. Crain, P. F., Yamamoto, H., McCloskey, J. A., Yamaizumi, Z., Nishimura, S., Limberg, K., Raba, M. & Gross, H. J. (1980) *Adv. Mass Spectrom.* **8**, 1135–1141.

21. Kimura-Harada, F., von Minden, D. L., McCloskey, J. A. & Nishimura, S. (1972) *Biochemistry* **11,** 3910–3915.
22. Barber, M., Bardoli, R. S., Sedgwick, R. D. & Tyler, A. N. (1981) *J. Chem. Soc. Chem. Commun.,* 325–327.
23. Kirsch, D. R., Wiebers, J. L. & Cohen, E. H. (1977) *J. Cell Biol.* **75,** Suppl. 131a.
24. Schoen, A. G., Cooks, R. G. & Wiebers, J. L. (1979) *Science* **203,** 1249–1251.
25. Wiebers, J. L. (1976) *Nucleic Acids Res.* **3,** 2959–2970.
26. Unger, S. E., Schoen, A. E., Cooks, R. G., Ashworth, D. J., Gomes, J. D. & Chang, C. (1981) *J. Org. Chem.* **46,** 4765–4773.
27. Nikolskaya, I. I., Lopatina, N. G. & Debov, S. S. (1976) *Biochim. Biophys. Acta* **435,** 206–210.
28. Kirnos, M. D., Khudyakov, I. Y., Alexandrushkina, N. I. & Vanyushin, B. F. (1977) *Nature (London)* **270,** 369–370.
29. Engel, J. D. & von Hippel, P. H. (1974) *Biochemistry* **13,** 4143–4158.
30. Engel, J. D. & von Hippel, P. H. (1978) *J. Biol. Chem.* **253,** 927–934.
31. Hattman, S., Goradia, M., Monaghan, C. & Bukhari, A. I. (1982) *Cold Spring Harbor Symp. Quant. Biol.* **47,** 647–653.
32. Plasterk, R. H. A., Vrieling, H. & Van de Putte, P. (1983) *Nature (London)* **301,** 344–347.