bon **22**, 969 (1980)]. Continued research at the site and on the methodologies of bone dating (H. Haas and J. Banewicz, *ibid.*, p. 537) may lead to reporting of accurate dates in the future.
4. H. B. Willman and J. C. Frye, *Ill. State Geol. Surv. Bull.* **94**, 79 (1970); C. V. Haynes, in *Prehistoric Man and His Environments: A Case Study in the Ozark Highlands*, W. R. Wood and R. B. McMillan, Eds. (Academic Press, New York, 1976), p. 56.
5. R. W. Graham, *Living Mus.* **42**, 43 (1980).
6. E. W. Haury, *Am. Antiq.* **19**, 11 (1953); ———, E. B. Sayles, W. W. Wosley, *ibid.* **25**, 2 (1959); C. V. Haynes, Jr., *Science* **145**, 1408 (1964).
7. J. L. Cotter, *Proc. Acad. Nat. Sci. Philadelphia* **89**, 12 (1937); E. W. Haury, *Am. Antiq.* **19**, 8 and 9, figures 6 and 7d (1953); J. M. Warnica, *ibid.* **31**, 350, figure 6G (1966).
8. E. Callahan, *Archaeol. East. North Am.* **7**, 15 (1979).
9. D. W. Dragoo, *ibid.* **1**, 13, figure 6h (1973).
10. J. E. King, *Ecol. Monogr.* **43**, 540 (1973); ——— and H. W. Allen, Jr., *Quat. Res. N.Y.* **8**, 321 (1977); P. A. Delcourt, H. R. Delcourt, R. C. Bristler, L. E. Lackey, *ibid.* **13**, 111 (1980); J. E. King, in *Archaic Prehistory on the Prairie Plains Border*, A. E. Johnson, Ed. (University of Kansas, Lawrence, 1980), p. 4; J. E. King, *Ecol. Monogr.* **51**, 43 (1981).
11. J. E. King and E. H. Lindsay, in *Prehistoric Man and His Environments: A Case Study in the Ozark Highlands*, W. R. Wood and R. B. McMillan, Eds. (Academic Press, New York, 1976), p. 75.
12. Supported by National Geographic Society research grant 2234-80 and funds from the Missouri Department of Natural Resources and the St. Louis Academy of Sciences. Specimens from the Field Museum of Natural History were loaned to us by G. Cole. We thank F. B. King and M. A. Graham for technical assistance. This is contribution No. 56 of the Archeological and Quaternary Studies Program of the Illinois State Museum.

20 February 1981; revised 1 June 1981

Polymorphism in the 5′-Flanking Region of the Human Insulin Gene and Its Possible Relation to Type 2 Diabetes

Abstract. *The arrangement of the human insulin gene in DNA from 87 individuals was analyzed by the Southern blot hybridization technique with a cloned genomic human insulin probe. Insertions of 1.5 to 3.4 kilobase pairs in the 5′-flanking region of the gene were found in DNA from 38 individuals. These insertions occurred within 1.3 kilobase pairs of the transcription initiation site. In contrast, no insertions were observed in the region 3′ to the coding sequence. The prevalence of these insertions in type 2 diabetes was significantly greater than in the other groups* ($P < .001$). *The limitation of this striking length polymorphism to a potential promoter region suggests that these insertions may play a role in insulin gene expression.*

Clinical diabetes includes a genetically heterogeneous group of disorders characterized by glucose intolerance (*1*). A higher concordance for diabetes in monozygotic versus dizygotic twins implicates genetic factors in the etiology of the disease, although the mode of inheritance is unknown. At present idiopathic diabetes mellitus has been divided into two major groups on the basis of family, twin, metabolic, immunologic, and HLA-association studies (*2*). Type 1 diabetes usually has its onset early in life, a strong association with certain HLA antigens, and a high prevalence of antibodies to islet cells. Type 2 diabetes generally develops in adults, bears no relation to distinct HLA haplotypes, and is not associated with antibodies to islet cells.

The two groups differ with regard to endogenous insulin production (*3*). In type 1 diabetes, because of destruction of pancreatic beta cells, there is absolute insulin deficiency, whereas in type 2 some ability to synthesize insulin is maintained. A relative insulin lack depends in part on the extent of coexistent insulin resistance (*4*). This feature is analogous to the deficient globin production in the various thalassemias (*5*) in which the α- and β-globin gene complexes give evidence of molecular defects. In the present studies we have examined the insulin gene in diabetics to determine whether alterations similar to those found in the thalassemias could be responsible for impaired insulin production.

Structural studies of the human insulin gene in diabetics have become possible as a result of recent advances in recombinant DNA technology. A total of 19 kb [kilobase (pairs)] of human DNA was cloned, and 1430 bp (base pairs) containing the insulin gene were sequenced (*6*). In addition, 5650 bp on the 5′ side and 11,500 bp on the 3′ side of the gene were analyzed by restriction endonuclease mapping. The entire region was shown to be single-copy DNA except for 500 bp of repetitive DNA located 6 kb 3′ to the gene (*6*). There is no evidence for more than one nonallelic insulin gene in humans (*6*). We have used the cloned human insulin gene probe to study the arrangement of the human insulin gene in type 1 and type 2 diabetics and compared it with the gene in nondiabetics by the Southern analysis (*7*) of DNA from 87 individuals.

High molecular weight DNA was prepared from peripheral blood leukocytes or human placental tissue (*8*). Leukocytes were isolated from 10 to 25 ml of whole blood by centrifugation at 500*g* at 22°C through a Ficoll-Hypaque gradient (*9*). In some instances, a crude nuclear fraction was obtained according to the method of Cordell *et al.* (*10*). Placenta obtained fresh at birth and stored at −70°C until use was pulverized on Dry Ice and lyophilized before DNA extraction. The DNA was purified essentially as described (*8*, *11*). After isolation, the DNA was precipitated with ethanol, dissolved in 10 m*M* tris, 1 m*M* EDTA, *p*H 7.5, and dialyzed extensively against 1 m*M* tris, 0.1 m*M* EDTA, *p*H 7.5, usually for 5 to 7 days. Typical yields included 200 to 400 μg of DNA per 10 ml of blood, and 1 to 2 mg of DNA per gram of placenta.

Restriction endonucleases were used according to the manufacturers' specifications (New England Biolabs and Bethesda Research), except that 2.5 to 3 units of enzyme were added to the reaction mixture per microgram of DNA. Completeness of digestion was monitored by adding 1 μg of lambda DNA to the reaction mixture. The samples were then subjected to electrophoresis through 0.8 to 1.5 percent agarose gels in

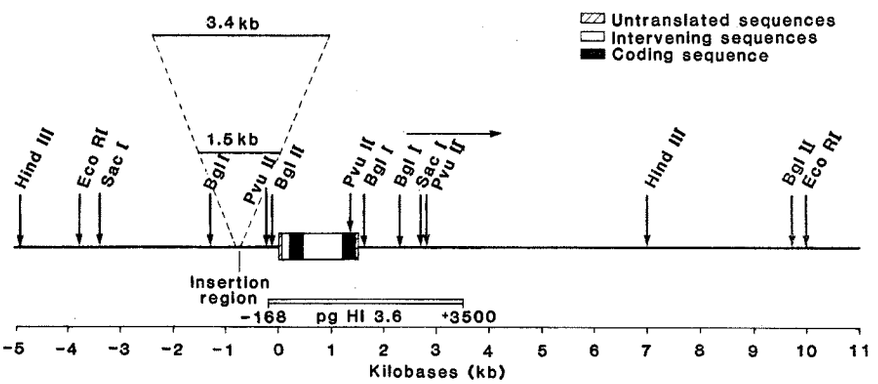

Fig. 1. Human insulin gene restriction map. The restriction sites were determined after single and multiple enzyme digests. The sites presented in this figure are only those used in our study. For a more complete map see (*6*). The insertion region falls somewhere between the Bgl I site (−1300) and Pvu II site (−259). The size of the insertions varied between 1.5 kb and 3.4 kb. The direction of transcription of the gene is shown by the arrow.

0036-8075/81/0904-1117$01.00/0

50 m*M* tris, 12 m*M* sodium acetate, 1 m*M* EDTA (*p*H 8) for 15 to 20 hours and transferred to nitrocellulose filters (BA85, Schleicher & Schuell) as described (*7*). Hybridization of the DNA-containing filters and subsequent steps were as described (*12*).

A 3.6-kb DNA fragment obtained by successive digestion with Bgl II and Xho I and spanning coordinates −168 to +3500 with respect to the putative transcription initiation site of the human insulin gene was isolated from recombinant pgHI 12.5 (*6*) (Fig. 1) and inserted into plasmid pBR322 by S1 endonuclease treatment and subsequent addition of Hind III linkers. The recombinant plasmid pgHI 3.6 was grown in *Escherichia coli* HB101. The insert was purified by digestion with Hind III and electrophoresis on 1 percent agarose gels. The 3.6-kb band was eluted by electrophoresis, extracted with phenol, and precipitated with ethanol. The DNA probe was labeled with ^{32}P by nick translation (*13*), generally to 2×10^8 to 3×10^8 counts per minute per microgram.

DNA from diabetic and nondiabetic subjects was studied by hybridization to pgHI 3.6. Digestion of genomic DNA with Eco RI generated a 14-kb hybridizing fragment (Fig. 2), which appeared to be the same size as that previously reported (*6*). The presence of a single 14-kb Eco RI fragment was observed in 17 nondiabetic, 8 type 1 diabetic, and 6 type 2 diabetic subjects (Table 1). In one nondiabetic and two type 2 diabetics, in addition to the 14-kb band, there was a hybridizing fragment of DNA approximately 15.5 kb in size. In another type 2 diabetic an extra band of 17.4 kb was observed. The presence of one normal fragment and one larger one in these four individuals suggested either that one allele contained an insertion of DNA between normal Eco RI sites or an altered Eco RI site.

If an insertion was present in the DNA of the four individuals with variant Eco RI patterns, we expected to also find additional larger hybridizing bands on analysis with other restriction endonucleases. Therefore, DNA from these subjects and others was digested with Hind III, Sac I, Bgl I, Bgl II, and Pvu II. Digestions with Hind III or Sac I in each case produced one normal DNA fragment and one larger, by approximately 1.5 kb in three of the subjects, and 3.4 kb in the fourth. In others digested with Hind III and Sac I comparable larger bands were found. This confirmed the presence of an insertion of DNA in one allele of these individuals, either within the insulin gene or its flanking regions.

Digestion of DNA from additional nondiabetics with Bgl I (Fig. 3) extended the number with length polymorphism to a total of 9 of 35 (Table 1). Bgl I fragments hybridizing with pgHI 3.6 included a 2.2-kb 3′ fragment and either a 2.9- or 3.5-kb 5′ fragment; the presence of two 5′ fragments is due to partial cutting at the middle Bgl I site at +1600 (see Fig. 1). The expected, smaller 0.6-kb fragment was not readily detected. All subjects had a 2.2-kb band, and at least one 2.9-kb band, or 2.9- and 3.5-kb band (Fig. 3, lanes 2 to 5). Those with length polymorphism in the second allele had additional hybridizing bands of 4.4 kb and 5.0 kb (Fig. 3, lane 4), 5.4 and 6.0 kb, or 6.2 and 6.8 kb (data not shown), representing an insertion of 1.5 kb, 2.5 kb, and 3.4 kb in one allele of the Bgl I 5′ fragment. In addition, another variant (N59) with Bgl I fragments of 4.4 and 5.0 plus 4.8 and 5.4 kb was seen (Fig. 3, lane

Table 1. Lengths (in kilobase pairs) of Eco RI and Bgl I fragments hybridizing to pgHI 3.6. DNA from 87 individuals was digested with Eco RI or Bgl I, subjected to electrophoresis, transferred, and hybridized to ^{32}P-labeled pgHI 3.6; the size of the hybridizing fragments was determined as described in the legend to Fig. 2. The Bgl I value refers only to the length of the 5′ fragment seen in the restriction map (Fig. 1). The size of the 3′ fragment was 2.2 kb in all individuals tested. In subjects where Eco RI hybridizing bands were not determined, comparable restriction fragments were obtained with either Hind III or Sac I. Insertions were present in 9 of 35 nondiabetics and in 6 of 17 type 1 and 23 of 35 type 2 diabetics. Chi-square analysis of the prevalence of insertions in type 2 diabetics compared to nondiabetics and type I diabetics indicates a statistically significant difference at $P < .001$. Additional length heterogeneity of approximately 0.2 kb was identified in 11 of the individual samples digested with Bgl I. This represents either a restriction site polymorphism or a deletion of DNA and was found in five nondiabetics and in four type 1 and two type 2 diabetics.

Individual	Eco RI	Bgl I	Insert (kb)	Individual	Eco RI	Bgl I	Insert (kb)
	Nondiabetic				*Type 1 diabetics*		
1	14/14	2.9/2.9		24		2.9/4.4	1.5
2	14/14	2.9/2.9		27		2.9/2.9	
6	14/14	2.9/2.9		28		2.9/2.9	
8	14/14	2.9/2.9		29		2.9/4.4	1.5
9	14/14	2.9/2.9		30		2.9/4.4	1.5
17	14/14	2.9/2.9		pl	14/14		
27		2.9/4.4	1.5	11JR3		2.7/4.4	1.5
28		2.9/4.4	1.5	J22		2.9/4.4	1.5
31	14/14	2.9/2.9			*Type 2 diabetics*		
32	14/14	2.9/2.9		2		2.9/4.4	1.5
34	14/14	2.9/2.9		3	14/14	2.9/2.7	
36	14/15.5	2.9/4.4	1.5	5		2.9/4.4	1.5
37		2.9/4.4	1.5	10		2.9/4.4	1.5
39	14/14			11	14/14	2.9/2.9	
40	14/14	2.9/2.9		19		2.9/4.4	1.5
41	14/14			20		2.9/2.9	
43		2.9/2.9		24		2.9/4.4	1.5
46		2.9/2.9		25		2.9/4.4	1.5
47		2.9/4.4	1.5	26	14/15.5	2.9/4.4	1.5
48		2.9/2.9		31		2.9/5.4	2.5
49		2.9/2.9		32	14/14	2.9/2.9	
51	14/14	2.7/2.7		33		2.9/4.4	1.5
53	14/14	2.9/2.7		43		2.9/2.9	
54	14/14	2.9/2.9		45	14/14		
56		2.9/2.7		46		2.9/2.9	
58	14/14	2.9/2.9		48		2.9/4.4	1.5
59		4.4/4.8	1.5/1.9	50		2.9/4.4	1.5
60		2.9/4.4	1.5	51		2.9/4.4	1.5
61		2.9/4.4	1.5	55		2.9/4.4	1.5
65		2.9/2.9		57		2.9/4.4	1.5
10p	14/14	2.9/2.9		58		2.9/4.4	1.5
15p		2.9/2.9		59		4.4/4.4	1.5
25J		2.9/2.7		61		2.9/4.4	1.5
26J		2.9/2.7		62		2.9/4.4	1.5
11JR5		2.9/4.4	1.5	63		2.9/2.9	
	Type 1 diabetics			64		2.9/2.7	
1	14/14	2.9/2.9		2p		2.9/2.9	
2		2.9/4.4	1.5	1p		2.9/4.4	1.5
3	14/14	2.9/2.7		4p	14/17.4	2.9/6.3	3.4
7		2.9/2.9		5p	14/14	2.9/2.9	
8	14/14			6p	14/15.5	2.9/4.4	1.5
11	14/14	2.9/2.7		8p	14/14	2.9/2.9	
13	14/14	2.9/2.9		11R2		2.9/4.4	1.5
14	14/14	2.9/2.9		11R4		4.4/4.4	1.5/1.5
18	14/14	2.9/2.7					

6). This represented a 1.5-kb insertion in one allele, and a 1.9-kb insertion in the other. The Bgl I digestion of 34 type 2 diabetics confirmed the length polymorphism observed with Eco RI in three subjects and extended the total number with insertions to 23 of 35.

The resolution in these methods is such that differences in length of less than 0.1 kb cannot be detected. In most individuals the presence of length polymorphism was independently determined by analysis with several restriction enzymes, thereby eliminating the possibility of artifact due to partial digestion. Digestion of the DNA from all individuals, including those with length polymorphism, with Bgl II or Pvu II produced identical fragments (Fig. 4). This indicated that the length polymorphism was due to an insertion of DNA somewhere between the Bgl I site at −1300, and the Pvu II site at −259, in the 5′ region flanking the insulin gene.

Variation in fragment length after digestion with restriction enzymes can arise from single base changes in DNA or from insertions or deletions. If this occurs in more than 2 percent of the individuals, the site (by definition) is a polymorphic locus (*14*). This type of genetic polymorphism has been used extensively in analysis of human globin genes. For example, polymorphism at an

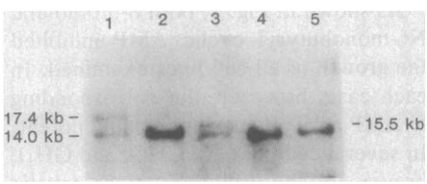

Fig. 2. A radioautograph of Eco RI–digested DNA hybridized with ^{32}P-labeled pgHI 3.6. The DNA from five individuals was isolated, digested with restriction endonucleases, and subjected to electrophoresis; the fragments were transferred to nitrocellulose filters and hybridized to ^{32}P-labeled pgHI 3.6 (*11*). The ^{32}P-labeled insulin gene probe (3×10^6 to 4×10^6 count/min) was added to each filter during hybridization. The filters were incubated for 20 to 24 hours at 42°C and washed, first briefly at room temperature in 2X SSC and 0.1 percent sodium dodecyl sulfate (SDS) (1X SSC is 0.15*M* sodium chloride and 0.015*M* sodium citrate, *p*H 7.0), then at 45°C in 0.2X SSC and 0.1 percent SDS. The filters were dried in air and autoradiographed at −70°C for 2 to 4 days (Kodak X-omat R x-ray film and a Dupont Lightning Plus intensifying screen). The size of the hybridizing fragments was determined by coelectrophoresis of Hind III and Eco RI–digested lambda DNA. Of the 50 individuals examined with Eco RI, most had a single 14-kb hybridizing fragment as depicted in lanes 2, 4, and 5. A 3.4-kb insertion can be seen in one allele of the DNA from the individual in lane 1, and a 1.5-kb insertion can be seen in one allele in lane 3.

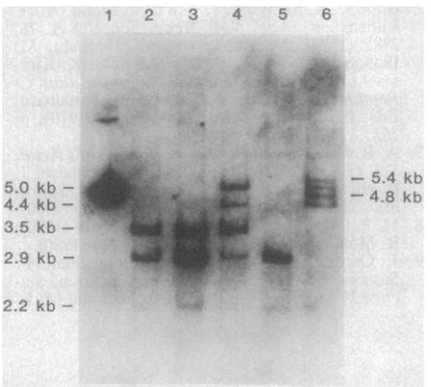

Fig. 3. Radioautographs of Bgl I–digested DNA hybridized with ^{32}P-labeled pgHI 3.6 (see Fig. 2). The data shown were obtained from five individuals (different from those of Fig. 1). Lane 1 contains an internal control consisting of a 4.8-kb preproinsulin (from rat) complementary DNA (500 pg) recombinant plasmid pcR1354. Lanes 2 to 6 contain 20 μg of DNA from each of five different individuals. Lanes 2, 3, and 5 represent the most commonly observed pattern and the difference between these is due to partial digestion at the Bgl I site at +1600 bp. In contrast, the patterns observed in lanes 4 and 6 indicate insertions of DNA.

Hpa I site 3′ to the β-globin gene is highly associated with the sickle cell gene mutation (*8*). Yet sequence variation around the β-globin gene in human individuals is quite low. Jeffreys found only two polymorphic sites when he screened more than 40 kb of β-globin genes from 60 humans with eight different enzymes (*15*). Comparable single base changes within the coding region of the insulin gene leading to a variant insulin molecule and impaired carbohydrate tolerance have been described in three individuals (*16*).

The type of length polymorphism in human DNA described in our report, resulting not from base substitutions but from insertions or deletions, has also been described (*17*). A polymorphic locus was found by using a single copy, human DNA probe which was not associated with any specific gene. Length polymorphism has also been observed between the embryonic and adult α-globin genes. The nature of these DNA insertional elements is unknown at present.

The DNA segments inserted near the human insulin gene may be transposable elements. Transposons in prokaryotes are pieces of DNA that vary in size, terminate in inverted or direct repeats, and that promote their own rapid spread within and between bacterial species (*18*). Similar eukaryotic transposable elements may be responsible for mutations in yeast and *Drosophila*, antibody diversity in mammals, and cellular transformation by RNA tumor viruses (*19*). However, transposable elements are characterized in general by the insertion of a constant piece of DNA at multiple sites, while the allelic variation which we observed adjacent to the human insulin gene was characterized by insertions of various length in a single region of the genome. This situation is more closely paralleled by the mating-type locus in yeast, where an insertion of DNA into the MAT locus determines the mating phenotype of the cell (*18*). It is not known at present whether the insertional elements noted in the 5′-flanking region of the human insulin gene are related to one another in sequence, are transposable genetic elements (that is, show repeated sequences), are stably inherited or are the result of somatic rearrangement, or all occur at precisely the same site. Clarification of these possibilities awaits the cloning and DNA sequence analysis of human insulin genes with 5′-flanking insertions.

The presence of insertional elements in the 5′-flanking region of the insulin gene raises the speculation that they may affect expression. A number of studies have suggested that DNA sequences several hundred bases 5′ to the mRNA transcription initiation site may modulate RNA polymerase binding and initiation of transcription (*18*). The presence of insertions or deletions in the 5′-flanking regions may alter DNA packaging within chromatin (*20*).

The importance of the above results is the common occurrence of insertions of DNA of variable size in a potential promoter region of the human insulin gene. Our data show the prevalence of insertions was 66 percent in those with type 2

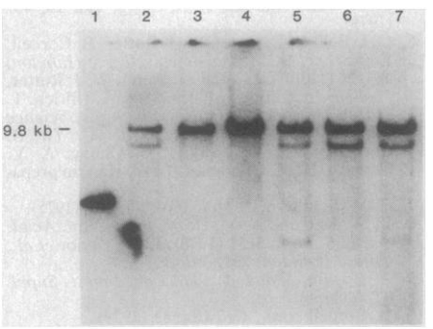

Fig. 4. A radioautograph of Bgl II–digested DNA from five individuals. The methods are those described in Fig. 2. In lane 1 is the 4.8-kb recombinant plasmid pcR1354 (500 pg). Lanes 2 to 7 contain 20 μg each of DNA from six individuals. In lanes 2, 5, 6, and 7, an additional internal control was added consisting of 20 to 50 pg of the human plasmid pgHI 3.6, which migrates as an 8.2-kb band. Lanes 5 and 7 also contain 20 pg of the 3.6-kb pgHI 3.6 insert.

diabetes and 29 percent in all others including nondiabetics and type 1 diabetics (Table 1). This difference is statistically significant at $P < .001$, although our findings do not indicate that these large insertional elements are sufficient for, nor necessarily associated with, development of diabetes. While this work was being done, similar length polymorphism in the 5′-flanking region of the human insulin gene was reported in 8 of 15 individuals studied by Bell *et al.* (*6*). Insertions of 100 to 2600 bp were noted, which were not restricted to diabetics (*21*). In view of the known complexity of the genetic factors contributing to type 2 diabetes (*1*), it is not surprising that our data do not show a simple relation between the presence of insertions and disease. However, our results support a contribution to the etiology of diabetes from insertions 5′ to the insulin gene.

PETER ROTWEIN, ROSE CHYN
JOHN CHIRGWIN
Departments of Medicine and
Anatomy, Washington University,
St. Louis, Missouri 63110
BARBARA CORDELL
HOWARD M. GOODMAN
Howard Hughes Medical Institute
Laboratory, Department of
Biochemistry and Biophysics,
University of California,
San Francisco 94143
M. ALAN PERMUTT
Department of Medicine,
Washington University

References and Notes

1. J. I. Rotter and D. L. Rimoin, *Am. J. Med.* **70**, 116 (1981).
2. National Diabetes Data Group International Workgroup, *Diabetes* **28**, 1039 (1979).
3. S. S. Fajans, in *Endocrinology*, L. J. DeGroot, Ed. (Grune & Stratton, New York, 1979), vol. 2, p. 1007.
4. O. G. Kolterman, J. Insel, M. Saekow, J. M. Olefsky, *J. Clin. Invest.* **65**, 1272 (1980).
5. D. J. Weatherall and J. B. Clegg, *Cell* **16**, 467 (1979).
6. G. I. Bell, R. L. Pictet, W. J. Rutter, B. Cordell, E. Tischer, H. M. Goodman, *Nature (London)* **284**, 26 (1980); G. I. Bell, R. Pictet, W. J. Rutter, *Nucleic Acids Res.* **8**, 4001 (1980); A. Ullrich, T. J. Dull, A. Gray, J. Brosius, I. Sures, *Science* **209**, 612 (1980); R. V. Lebo, L. C. Yu, B. Cordell, M.-C. Chalving, J. C. Chong, A. V. Carrano, H. M. Goodman, Y. W. Kan, in preparation.
7. E. M. Southern, *J. Mol. Biol.* **98**, 503 (1975).
8. Y. W. Kan and A. M. Dozy, *Proc. Natl. Acad. Sci. U.S.A.* **75**, 5631 (1978); J. M. Taylor *et al.*, *Nature (London)* **251**, 392 (1974).
9. A. Boyum, *Scand. J. Clin. Lab. Invest. Suppl.* **97**, 6 (1968).
10. B. Cordell *et al.*, *Cell* **18**, 533 (1979).
11. We thank Gary Temple in Y. W. Kan's laboratory for furnishing a detailed protocol of the procedure.
12. G. M. Wahl, M. Stern, G. R. Stark, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 3683 (1979).
13. P. W. J. Rigby, M. Dieckmann, C. Rhodes, P. Berg, *J. Mol. Biol.* **113**, 237 (1977).
14. H. Harris and D. A. Hopkinson, *Hum. Genet.* **36**, 9 (1972).
15. A. J. Jeffreys, *Cell* **18**, 1 (1979).
16. S. C. M. Kwok, S. J. Chan, A. H. Rubenstein, R. Poucher, D. F. Steiner, *Biochem. Biophys. Res. Commun.* **98**, 844 (1981); B. D. Given *et al.*, *N. Engl. J. Med.* **302**, 129 (1980); H. Tager *et al.*, *Nature (London)* **281**, 122 (1979); K. H. Gabbay, R. M. Wolff, J. Wolff, M. Mako, A. H. Rubenstein, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 2881 (1979); Y. Kanazawa, M. Hoyashi, M. Ikeuchi, M. Kasuga, Y. Oka, H. Sato, K. Kiramatsu, K. Kosaka, in *Proinsulin, Insulin, C-Peptide*, B. Shigeaki, T. Kaneko, N. Yanaihara, Eds. (Excerpta Medica, Amsterdam, 1979), p. 262.
17. A. R. Wyman and R. White, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 6754 (1980); J. N. Strathern, J. B. Hicks, A. J. S. Klar, K. Nasmyth, *J. Supramol. Struct. Cell. Biochem. Suppl.* **5**, 399 (1980).
18. M. P. Calos and J. H. Miller, *Cell* **20**, 579 (1980); R. McKay, *Nature (London)* **287**, 188 (1980).
19. J. Corden, B. Wasylyk, A. Buchwalder, P. Sassone-Corsi, C. Kedinger, P. Chambon, *Science* **209**, 1406 (1980); R. Grosschedl and M. L. Birnstiel, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 1432 (1980); L. H. T. Van der Ploeg, A. Konings, M. Oort, D. Roos, L. Bernini, R. A. Flavell, *Nature (London)* **283**, 637 (1980).
20. C. Wu and W. Gilbert, *Proc. Natl. Acad. Sci. U.S.A.* **78**, 1577 (1981).
21. G. Bell, personal communication.
22. We thank the Washington University Diabetes Research and Training Center and Drs. William C. Knowler and Barbara Howard for blood samples used in DNA preparation. Supported by NIH grant AM-16724, National Research Service Award AM-07120 (to P.R.), PHS career development award AM-00033 (to M.A.P.), and NIH grant CA-14026 (to H.M.G.).

8 May 1981; revised 24 June 1981

Growth Inhibition by Adenosine 3′,5′-Monophosphate Derivatives Does Not Require 3′,5′ Phosphodiester Linkage

Abstract. *Analogs of adenosine 3′,5′-monophosphate (cyclic AMP) inhibit the growth of cultured cell lines. The effects of 8-bromo- and N6-butyryl-substituted analogs of cyclic and noncyclic AMP on six cell lines were examined and were equally inhibitory. Variant cell lines with altered cyclic AMP-dependent protein kinase were more resistant to both cyclic and noncyclic nucleotides. We conclude that growth inhibition by analogs of cyclic AMP (i) does not require a 3′,5′ phosphodiester bond and (ii) may be mediated by a pathway involving endogenous cyclic AMP.*

Ryan and Heidrick (*1*) and Burk (*2*) reported that theophylline, a cyclic nucleotide phosphodiesterase inhibitor, and adenosine 3′,5′-monophosphate (cyclic AMP) inhibited the growth of BHK and HeLa cells, respectively. Subsequent studies with a wide variety of cultured cells demonstrated growth inhibition by cyclic AMP derivatives (*3, 4*). Such studies may indicate a role for cyclic AMP as a negative regulator of cell proliferation; however, growth stimulatory actions of cyclic nucleotides have also been reported (*5, 6*). In addition, studies with adenylate cyclase–deficient or protein kinase–deficient cell lines suggest a nonobligatory role for the nucleotide (*7*). Studies of the effects of cyclic AMP derivatives on growth are difficult to interpret because of the well-recognized biological potency of catabolites derived from the nucleotides in culture systems.

In previous studies (*8*) with cultured pituitary cells we reported that bromo and butyryl analogs of cyclic AMP inhibited growth by two distinguishable mechanisms. At low concentrations, adenosine derivatives were generated that resulted in cytotoxicity in adenosine-sensitive cells because of pyrimidine starvation. At higher concentrations in adenosine-resistant, adenosine kinase–deficient cell lines, both cyclic (3′,5′) and noncyclic (5′) AMP derivatives were growth inhibitory. In the studies reported here, we demonstrate that growth inhibition by cyclic AMP derivatives in a wide variety of cell lines does not require the 3′,5′ phosphodiester linkage. These results indicate that long-term effects of exogenous cyclic AMP derivatives are exerted by an unanticipated mechanism. However, additional studies also indicate that nucleotide-induced growth inhibition may involve both adenylate cyclase and cyclic AMP–dependent protein kinase.

As shown in Fig. 1, both 8-bromo and N6-monobutyryl cyclic AMP inhibited the growth of all cell lines examined. In each case, however, the corresponding 5′ AMP derivatives were equally potent. In several cell lines (L, CHO, and GH_1), bromo and butyryl derivatives (cyclic and noncyclic) were inhibitory over the same concentration range. In contrast, with BHK, BALB/c, and Y1 cells, 8-bromo nucleotides were more inhibitory than N6-monobutyryl nucleotides. Equipotency of cyclic and noncyclic derivatives was also observed with mono-, di-, and tributyryl nucleotides with 3T3 cells (not shown).

Noncyclic and cyclic AMP derivatives are extensively converted to adenosine derivatives during cell culture. Adenosine is cytotoxic to cultured cells as the result of inhibition of pyrimidine nucleotide biosynthesis (*9*). Growth inhibition reported in these studies cannot be mediated by such a pathway since uridine failed to prevent growth inhibition. Furthermore, similar growth inhibition by nucleotides is observed with adenosine-resistant, adenosine kinase–deficient cell lines (GH_1 and Y1; Fig. 1) (*10*).

The phosphodiesterase inhibitor the-

0036-8075/81/0904-1120$01.00/0