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THE MOLECULAR BIOLOGY OF ENVIRONMENTAL AROMATIC HYDROCARBONS

FINAL PROGRESS REPORT
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NOTICE

FINAL PROGRESS REPORT

B. Abstract

The induction of mutations in living cells by polycyclic aromatic hydrocarbons (PAH) has been recognized for many years. Although the mechanism for this occurrence has been examined by numerous investigators, the precise nature and type of mutations induced is still unclear. Earlier investigations of DNA damage and repair were primarily examined by the random alkylation of bacterial and mammalian DNAs, *in vivo*, using a variety of different PAH agents. This procedure is still used today. Though informative, such studies have not offered any explanation of the mechanism by which PAH agents induce carcinogenesis.

We have attempted to examine the repair of PAH-damaged DNA using small DNA oligomer constructs as targets for site-specific alkylation. DNA constructs containing a single BPDE alkylated site in each duplex strand were ligated into M13 RF DNA and used to transfect *E. coli*. Progeny M13 DNA was isolated from *E. coli* colonies grown on agar plates containing IPTG and Xgal. DNA sequence analysis of the isolated progeny M13 DNA, at the site of construct insertion, was found to contain large deletions and illegitimate recombinants. These sequence rearrangements occurred in either *rec*A$^+$ or *rec*A$^-$ host cells suggesting that SOS processing was not involved in the deletions and the recombinants observed. The mechanism by which BPDE induces illegitimate recombinants has not been resolved, however, it is possible that the closely spaced adducts activate the recombinant machinery in our DNA-damaged cells.

In collaboration with Dr. Ronald Harvey at this university, a chemical procedure has been developed for the synthesis of 6-(1-methylpyrenyl)-deoxyguanosine and 6-(1-methylpyrenyl)deoxyadenosine. After conversion to phosphoramidites, these single nucleoside PAH-derivatives can be site-specifically introduced into plasmid vectors without altering DNA nucleotide sequences. The use of prealkylated nucleotides in preparing oligomer constructs avoids random alkylation of DNA by activated PAH and the assembly of pallindromic constructs as employed in our previous experiments.

B. Introduction

It is widely recognized that certain chemical and physical agents are carcinogenic for a variety of living organisms. Present evidence suggest that damage of cellular DNA by these toxic agents which cause alterations in DNA sequence arrangement is primarily responsible for the induction of carcinogenesis. However, the precise mechanism by which cells are transformed from normal to a malignant phenotype has not been completely resolved.

Chemical agents such as polycyclic aromatic hydrocarbons (PAH) require metabolic activation to exert their mutagenic and carcinogenic effects. *In vivo*, metabolic activation is catalyzed primarily by the mixed function oxygenase enzymes of microsomes and diolepoxide derivatives of PAH have been implicated as the ultimate carcinogenic product for benzo[a]pyrene (BP) and other carcinogenic hydrocarbons. While activated hydrocarbons may interact with different cellular molecules, covalent binding to DNA is considered to be the most critical target in living cells.

In vivo, the active metabolite of benzo[a]pyrene which binds covalently to DNA and RNA has been shown to be benzo[a]pyrene-7,8-dihydrodiol-9,10-expoxide (BPDE). Both *anti-* and *syn*-BPDE are formed *in vivo*, however, anti-BPDE is more prevalent and more potent as a transforming agent for living cells. Elucidation of the mechanism by which BPDE induces mutagenesis is important since it involves modification of DNA sequences and DNA expression as is the case for malignant transformation of cells.

1. Experimental observations

a. Mutations induced by BPDE

In *Escherichia coli*, the inhibition of phage reproduction by different PAH compounds was first described in our laboratory (1,2). This inhibition was subsequently shown to result from the covalent binding of activated PAH (BPDE) to phage nucleic acid and that a single BPDE adduct was sufficient to prevent the replication of either phage DNA or phage RNA (3,4). Similar studies using monkey kidney cells transfected with SV40 DNA also showed marked inhibition of viral DNA replication by BPDE, however, this inhibition did not follow a single-hit mechanism and required multiple alkylation of DNA adducts (5,6). Examination of progeny SV40 DNA isolated from transfected cells indicated that a large percentage were defective, *i.e.*, noninfectious, which we speculated was caused by BPDE-induced mutations. To facilitate the detection of mutations, a relatively simple procedure was devised to identify sequence rearrangements induced by activated PAH agents.

In 1984, we reported the use of a model system for detecting mutations induced by BPDE at a small localized region in viral DNAs (7). M13 RF DNA was modified to contain a 10-base-pair Bam H1 linker alkylated with BPDE and the modified DNA was used to transfect *E. coli.*

After plating on agar containing IPTG and Xgal, colorless infected clones were selected, amplified, and viral DNAs were isolated for sequence analysis at the site of construct insertion by the Sanger dideoxy method. Using this technique, we observed that a significant number of progeny viral DNAs contained deletions ranging from 1-24 base pairs in length which included both target and nontarget nucleotide residues. Since the Bam H1 linker contains several different nucleotide residues that can bind covalently with BPDE, it was not possible to determine whether any one specific adduct was responsible for the deletions observed.

In our recent grant period, the above observation was pursued in greater depth. Duplex oligomer constructs were prepared containing a single BPDE adduct at a specific site in each of the two duplex strands and with different spacings between the two adducts (Figs. 1 and 2). These constructs were ligated into M13mp19 RF DNA and used to transfect *E. coli* JM105 cells which were plated on agar. After incubation for 24 hours at 37°C, progeny viral DNA isolated from selected colorless colonies were examined for sequence rearrangement by DNA sequence analysis. The results from these experiments were published in 1988 (8) and are summarized below.

M13 RF DNA modified to contain nonalkylated constructs, or constructs having a single BPDE adduct in one strand only, showed no alterations of DNA sequences. Constructs that contained a single BPDE adduct (dA or dG) in each strand of the oligomer duplex induced two kinds of sequence alterations, either large deletions or illegitimate recombinants both of which resulted in the removal of the damaged construct (Figs. 3, 4 and Table 1). These DNA sequence rearrangements were observed in both *rec*A$^+$ and *rec*A$^-$ *E. coli* host cells.

In *E. coli*, most mutations induced by ultraviolet light and chemical mutagens are SOS dependent, and each agent appears to cause its own characteristic spectrum of mutations. The *rec*A gene is an important component of the SOS processing system which is involved in the repair of DNA damaged by the above agents. Our findings that the deletions and recombinant events induced by BPDE are not dependent upon *rec*A function suggest that SOS processing is not involved in these sequence rearrangements. We have proposed that in our system it is not the specific lesion that is recognized for repair, but rather the rare distortion of DNA secondary structure created by the closely-spaced adducts which activate the recombinant machinery. If this hypothesis is correct, agents other than bulky PAHs that damage and distort DNA structure should also induce similar DNA sequence rearrangement.

b. DNA damage studies using ultraviolet light

To test the above hypothesis, we decided to use ultraviolet irradiation as the damaging agent for synthetically prepared oligomer constructs. UV irradiation does not involve covalent alkylation of nucleotide residues in DNA. We prepared 32-base-pair duplex constructs containing a single pyrimidine-pyrimidine dimer at a specific single site in each of the two duplex strands. Two single-stranded 15-mers were synthesized: (a) 5' TACGCACGATTAAAG 3', and (b) 5'

TACGCACGATTCAAG 3'. When exposed to UV irradiation, only a single TT dimer forms with oligomer (a), whereas with oligomer (b) formation of two different dimers are possible, *i.e.*, TT or TC(6-4) lesion. UV-damaged oligomers were separated from undamaged oligomers on 20% PAGE; the former migrates more slowly than the latter. Purified damaged and undamaged 15-mers were separately used to construct a 32 bp duplex by hybridization to complementary DNA oligomers followed by self-ligation as described for the preparation of BPDE-alkylated constructs (Fig. 1).

The 32 bp duplex constructs, damaged or undamaged, were ligated into M13 RF DNA and used to transfect *E. coli* host cells. Progeny viral DNAs were isolated and examined for sequence alterations as described for our studies with BPDE-damaged constructs. Growth of transfected cells on agar plates was conducted under conditions of darkness and in light. This procedure was used to distinguish between DNA sequence rearrangements induced by TT dimers and those dimers that are (6-4) photoproducts. Under conditions of light, *E. coli* DNA photolyase can reverse TT dimers by photoreactivation, but not (6-4) photoproducts.

Examination of progeny viral DNAs, independently isolated from several hundred colonies generated by the transfection of *E. coli* with M13 RF DNA containing UV-damaged constructs, was found to induce a very low frequency of deletions and illegitimate recombinants, (less than 1%). At the present time, therefore, we are uncertain whether this low frequency of recombinant induction is meaningful or perhaps may be an artifact of our experimental system. Further studies to resolve this problem are in progress.

3. Preparation of site-specific alkylated oligomers

It has long been recognized that the one consistent feature of malignant cells is the presence of aberrant chromosomes. The appearance of such chromosomal abnormalities in living cells might possibly be related to the repair of damaged DNA by different mechanisms including illegitimate recombinations. Recent studies in our laboratory have been directed towards exploring this possibility. Our initial objective has been to determine if mammalian cells utilize illegitimate recombinations in the process of repairing damaged DNA. To answer this question, we felt that it might be possible to use a model system similar to the system used in our *E. coli* studies. However, since the cells and DNA vector employed in mammalian studies would be different from those used in our *E. coli*/M13 experiments, certain modifications in our procedure would be necessary. Our current plan is to transfect a known mammalian cell line, *e.g.*, COS cells, with pSV2-gpt plasmid DNA modified to contain a duplex construct alkylated at a specific site in each strand. It was decided not to use the pallindromic construct employed in our *E. coli*/M13 system, but rather to prepare an alkylated construct which upon insertion into pSV2-gpt would not alter the nucleotide sequence arrangement of the plasmid vector.

In collaboration with Dr. Ronald Harvey at this university, a synthetic method was devised for the synthesis of 2-(1-methylpyrenyl)deoxyguanosine and 6-(1-methylpyrenyl)deoxyadenosine (Figs. 5 and 6). The chemical procedure involved for the synthesis of these alkylated nucleosides required extensive trial and error over a period of 1.5 years. The synthetically prepared PAH-nucleoside is modified to contain phosphoramidites at the 3'-OH group and blocked by reaction with dimethoxychloride. DNA oligomers are synthesized by the stepwise addition of normal (nonalkylated) nucleoside phosphoramidites (Agarwal, K.D. and Riftina, F., *Nucleic Acid Res. 8*, 2809, 1978) and a single PAH-nucleoside phosphoramidite is introduced into the growing chain at a selected site. Further extension of the chain to a predetermined length is accomplished by continued reaction with normal nucleoside phosphoramidite derivatives. Hence, this procedure allows the assembly of DNA oligomers containing a single, or multiple, site-specific adduct and the oligomer sequence can be made identical to a specified region of a chosen DNA vector. This specified region in the DNA vector can be removed by appropriate restriction enzymes and replaced by ligation of the vector with the synthetic oligomer construct containing the site-specific adduct. Thus, the exchange of vector DNA sequences for the synthetic construct described above does not alter the nucleotide sequence arrangement in the DNA vector. This modified method for studying mutations induced by chemical agents avoids the random alkylation of cellular or plasmid DNAs by activated PAHs as well as the use of pallindromic constructs employed in our previous experiments (8). At the present time, duplex constructs are in preparation by the above procedure. These constructs will be inserted into small viral vectors and used to transfect mammalian and bacterial cells. Progeny viral DNAs will be collected from single clones, amplified, and examined for mutations induced by the site-specific damaged constructs.

C. Selected Publications:

1. Hsu WT, Moohr JW, Weiss SB: (1965) Proc. Natl. Acad, Sci. U.S.A. **53**, 517.
2. Hsu WT, Moohr JW, Tsai AYM, Weiss SB: (1966) Proc. Natl. Acad. Sci. U.S.A. **55**, 1475.
3. Hsu WT, Lin EJ, Harvey RG, Weiss SB: (1977) Proc. Natl. Acad. Sci. U.S.A. **74**, 3335.
4. Sagher D, Harvey RG, Hsu WT, Weiss SB: (1979) Proc. Natl. Acad. Sci. U.S.A. **76**, 620.
5. Chang GT, Harvey RG, Hsu WT, Weiss SB: (1979) Biochem. Biophys. Res. Commun. **88**, 688.

6. Chang GT, Harvey RG, Weiss SB: (1981) Biochem, Biophys. Res. Commun. **100**, 1981.
7. Wei SJC, Desai SM, Harvey RG, Weiss SB: (1984) Proc. Natl. Acad. Sci. U.S.A. **81**, 5936.
8. Kokontis JM, Vaughan J, Harvey RG, Weiss SB: (1988) Proc. Natl. Acad. Sci. U.S.A. **85**, 1043.

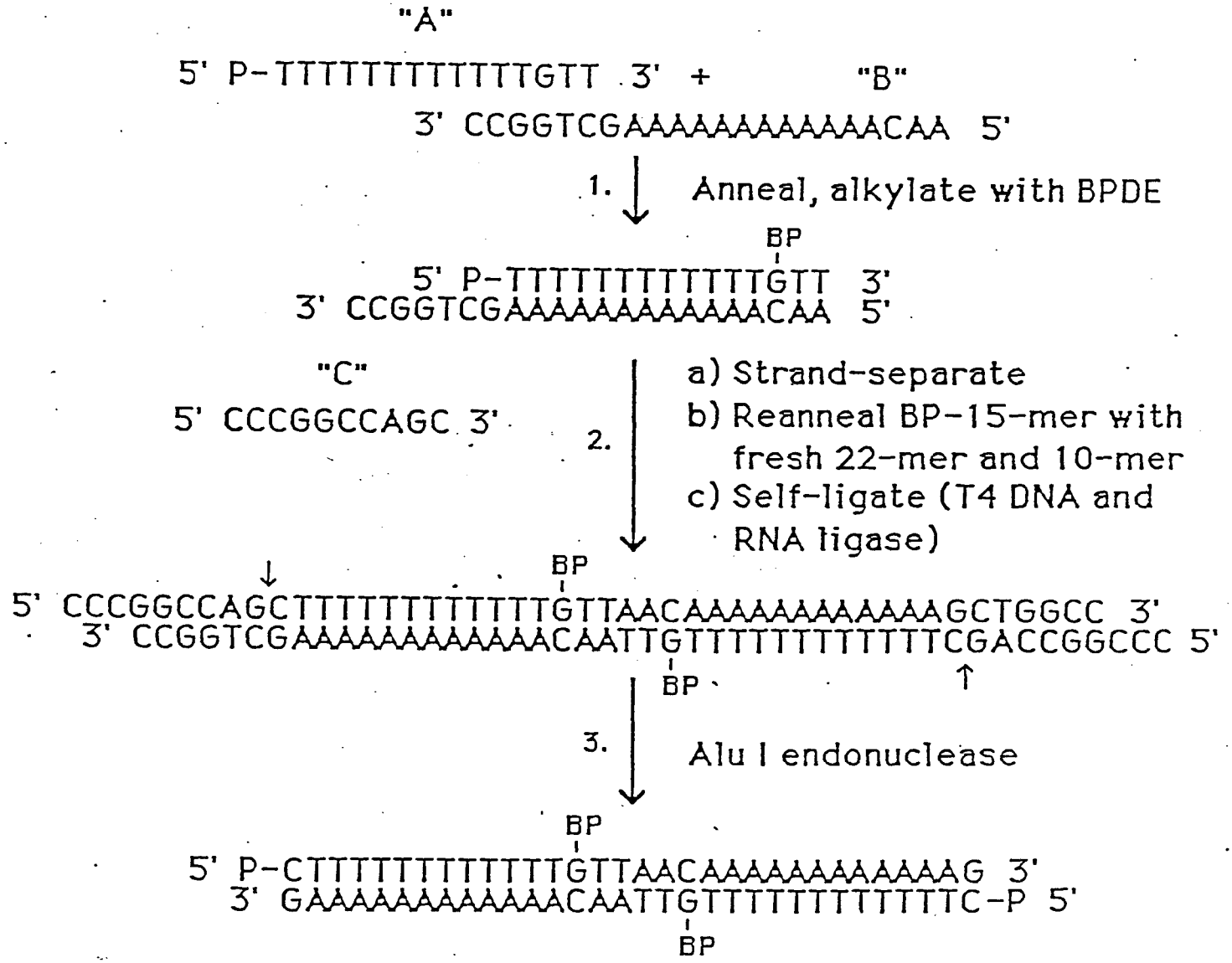

Figure 1. Scheme for the preparation of doubly alkylated 32-mer duplex.

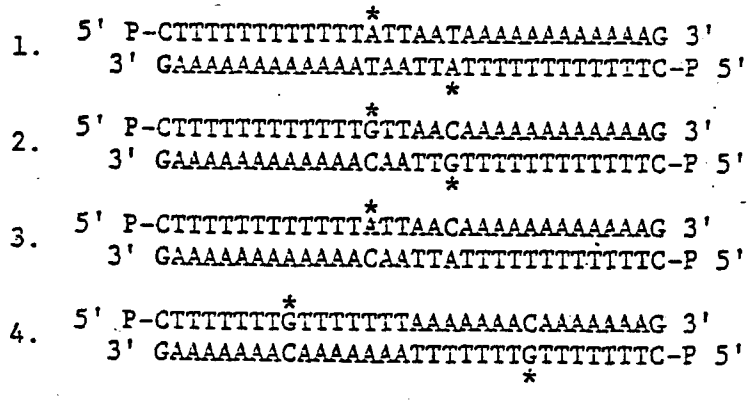

Figure 2. DNA oligomer constructs prepared. Of the 32-bp constructs used , some were nonalkylated and some were alkylated with BPDE at a specific single site (asterisk) in each strand, as shown. In the alkylated form, constructs 1 and 2 contained two BP adducts separated by 4 bp, construct 3 contained one BP adduct, and construct 4 contained two BP adducts separated by 14 bp.

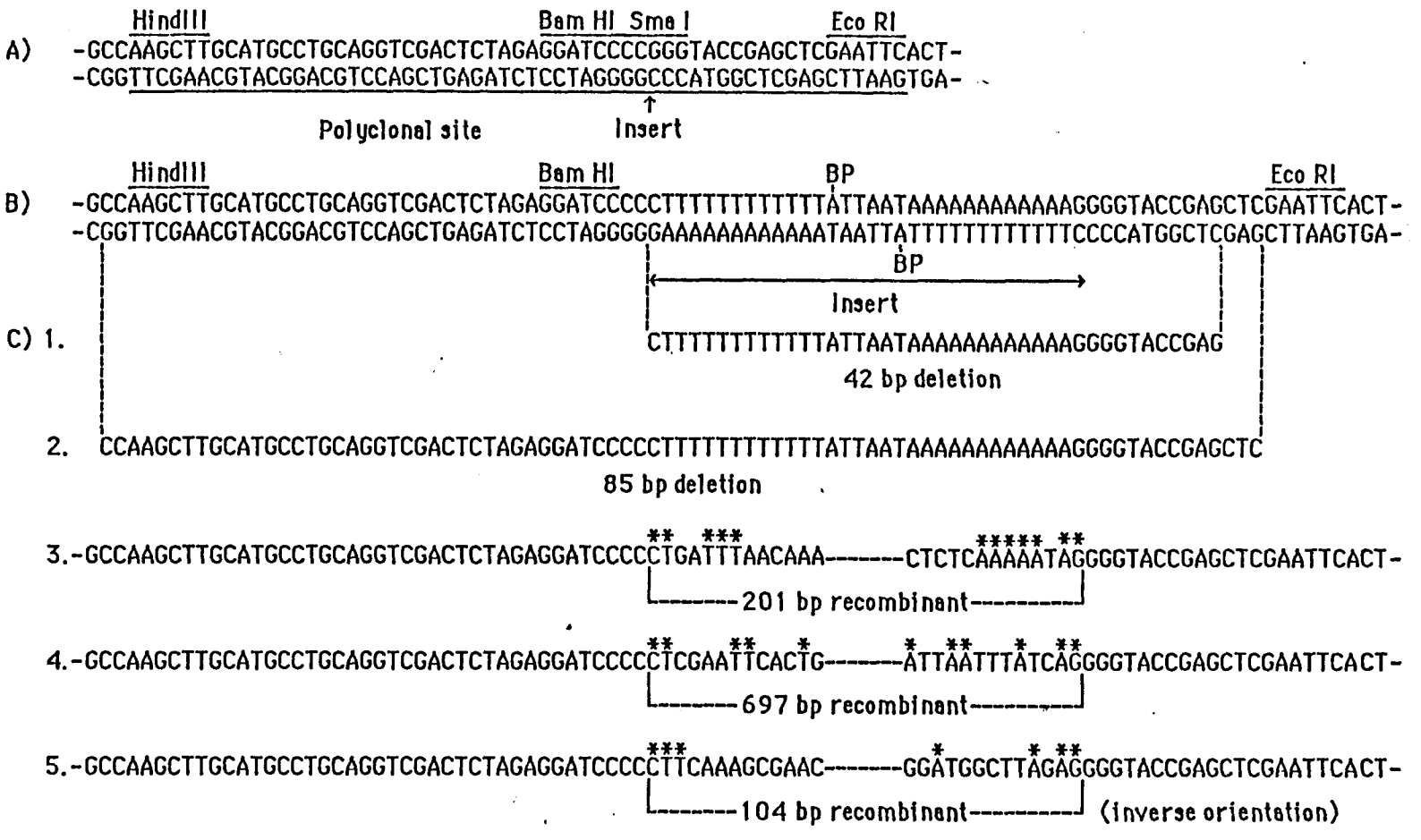

Figure 3. M13mp19 sequences modified by BP-alkylated constructs. (A) M13mp19 RF sequences at the polyclonal site. (B) BP construct inserted at the Sma I site of sequence in A. (C) Deletion and recombinant DNA sites in progeny single-stranded mp19. Recombinant bases identical with construct bases are indicated by asterisks.

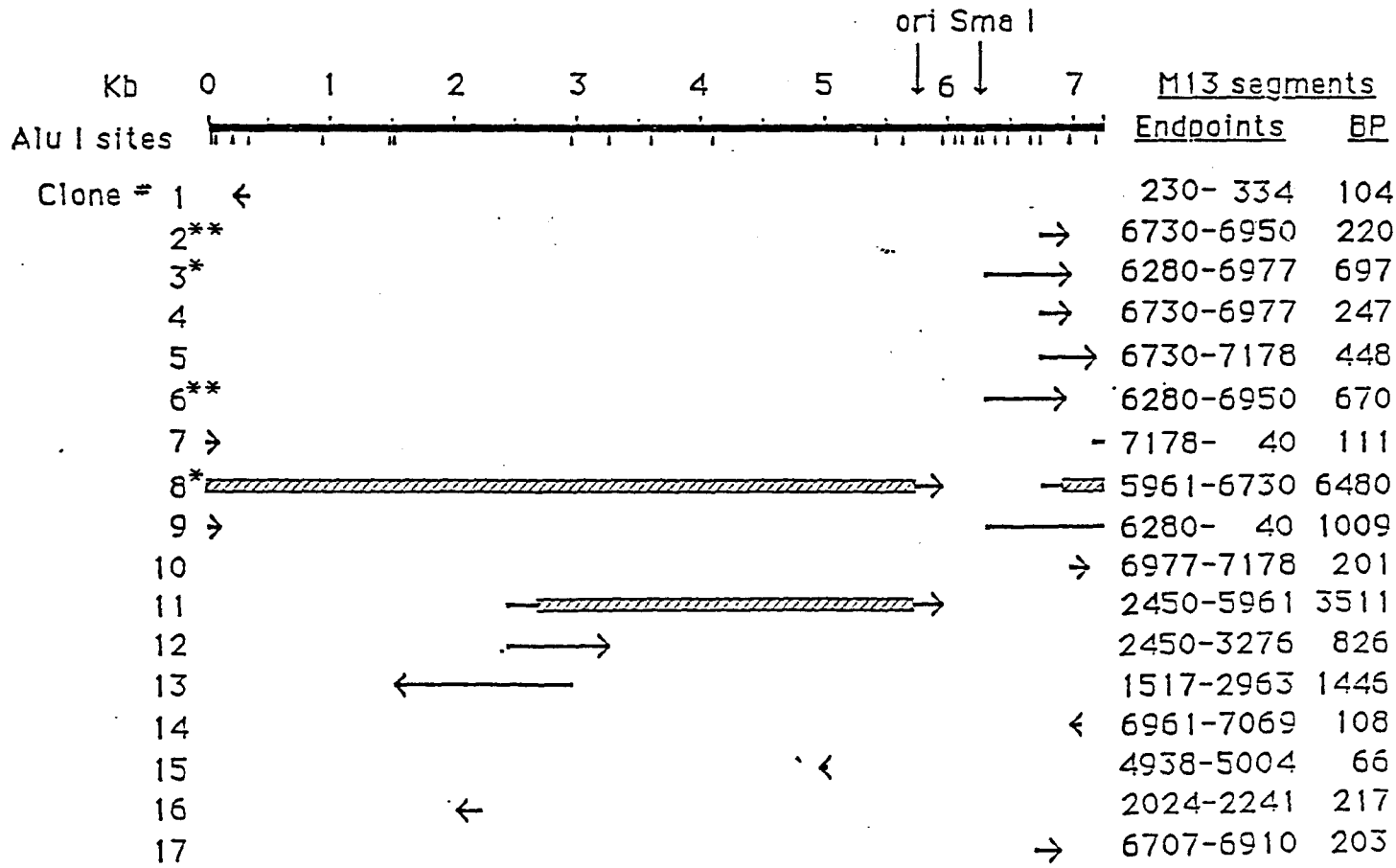

Figure 4. Origin and size of recombinant DNA segments. The heavy bar at the top represents the linear form of MI3mpl9 DNA. Vertical lines above and below this bar indicate the length (in kb) and Alu I sites, respectively. Horizontal lines mark the location and length of the recombinant segments in MI3 DNA, and the arrows show the segment orientation at the recombinant site. Hatched bars indicate the portion of a recombinant segment that was removed prior to packaging. The precise location and size of each recombinant, obtained from the terminal sequences as described in Fig. 3, are given in the two columns at the right. Asterisks indicate the number of additional clones found that had the same recombinant segment.

Table 1. Sequence alterations in progeny M13 DNA induced by BP-alkylated constructs

Construct[a] number	Construct[a] treatment	Host[b] cell	Total[c] clones	Sequence alterations		
				None	Deletions	Recombinants
1 and 2	None	JM105	294	294	0	0
3	BPDE 1x	JM105	27	27	0	0
3	BPDE 1x	JM109	12	12	0	0
1	BPDE 2x	JM105	20	1	6	13
2	BPDE 2x	JM105	19	7	8	4
2	BPDE 2x	JM109	13	3	5	5
4	BPDE 2x	JM105	17	9	7	1

The construct number and treatment with BPDE are as shown in Fig. 2. Constructs with 1 or 2 BP adducts are indicated as BPDE 1x or BPDE 2x, respectively. Host cell JM105 is *recA*$^+$; *gyrA*$^+$; JM109 is *recA1*$^-$ *gyrA96*$^-$. The complete genotype of both strains is given in Yanisch-Perron, C., Vieira, J. & Messing,J. (1985) Gene 33, 103-119. If the number of colorless plaques derived from nonalkylated construct inserts per ng of transfected DNA is taken as 100% survival, then the average survival of RF DNAs containing BP constructs in JM105 and JM109 is 1.5% and 0.8%, respectively.

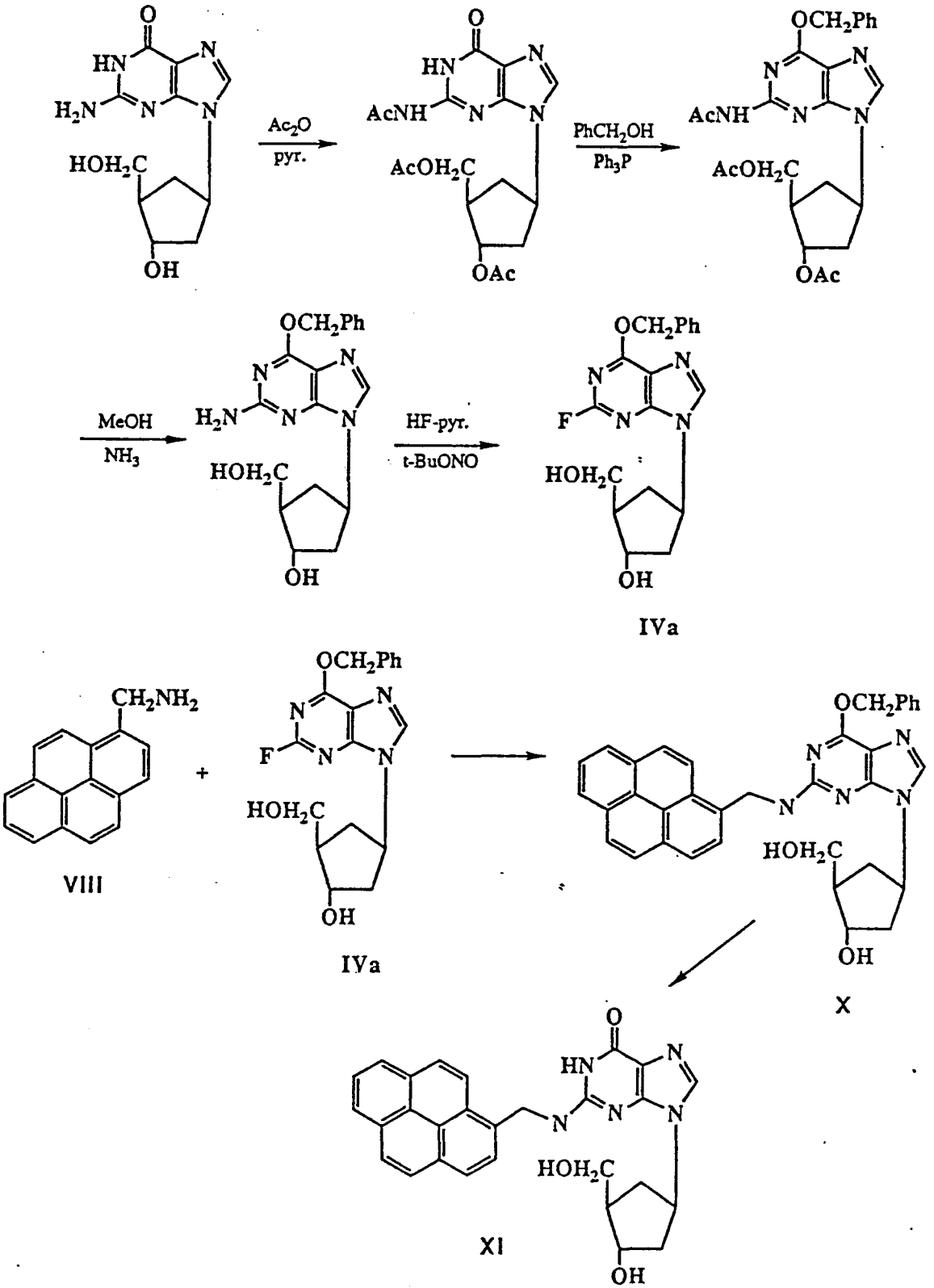

Figure 5 Synthesis of 2-(1-methylpyrenyl)guanosine by Method II.

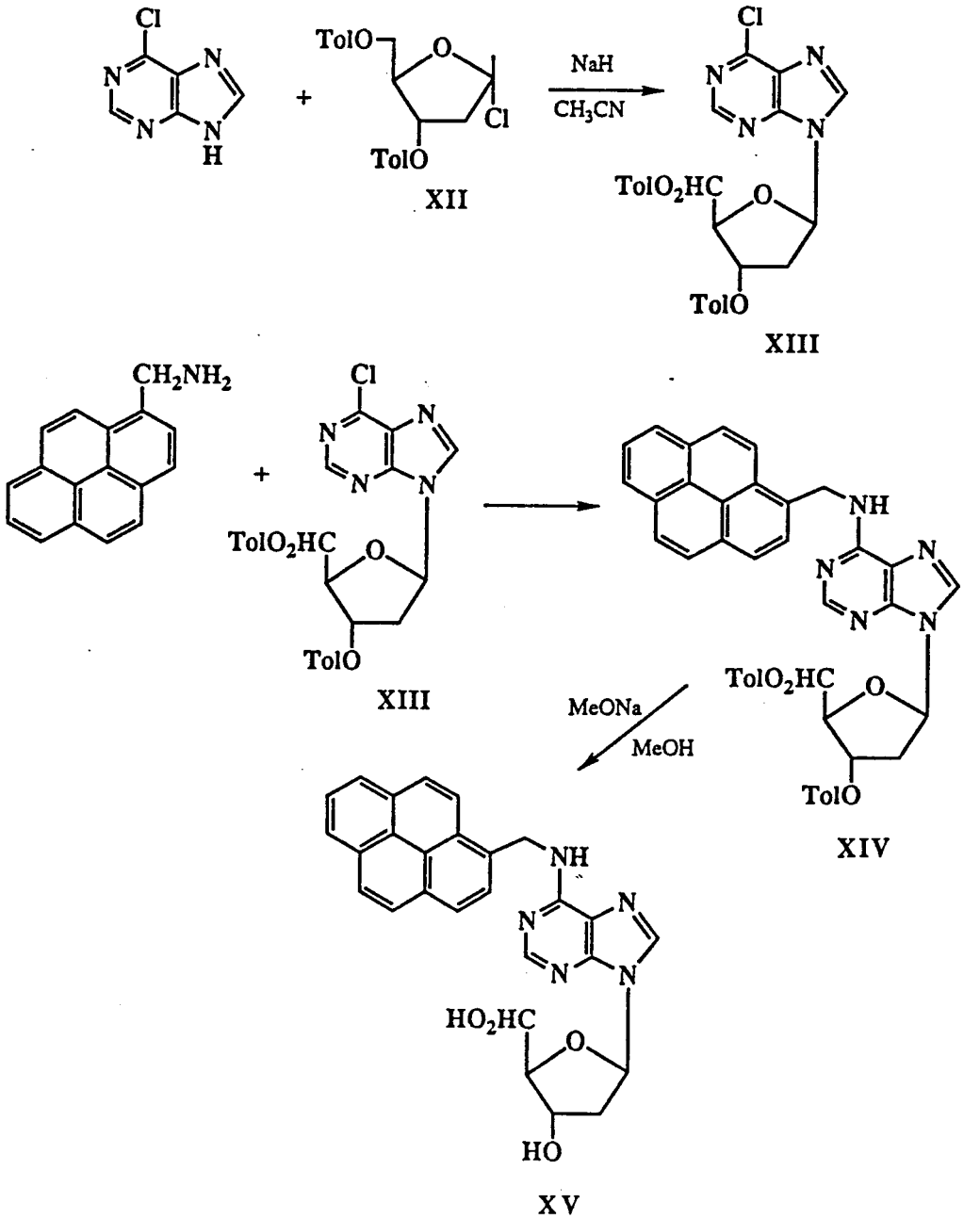

Figure 6. Synthesis of 6-(1-methylpyrenyl)adenosine by Method II.

LIST OF PUBLICATIONS

Kokontis, J.M., Vaughan, J., Harvey, R.G. and Weiss, S.B. (1988) Illegitimate recombination induced by benzo[a]pyrene diol epoxide in *Escherichia coli*. Proc. Natl. Acad. Sci. U.S.A. **85**:1043.