Base Pairing and Template Specificity during Deoxyribonucleic Acid Repair Synthesis in Human and Mouse Cells†

Michael W. Lieberman*,‡ and Miriam C. Poirier

ABSTRACT: Nucleotide insertion during DNA repair synthesis has been studied in cultured human diploid fibroblasts (WI-38) and Balb/c 3T3 mouse cells damaged with ultraviolet radiation and two mutagenic carcinogens, *N*-acetoxy-2-acetylaminofluorene (NA-AAF) and 7-bromomethylbenz[*a*]anthracene (7BrMeBA). Evidence from thermal elution chromatography, S_1 nuclease digestion studies, and pyrimidine isostich analysis suggests that in mammalian cells the complementary, undamaged strand serves as a template for repair synthesis and that the result of such synthesis is the accurate restoration of the damaged area to the original nucleotide sequence.

It is now widely recognized that many mammalian cells (*e.g.*, Legator and Flamm, 1973; Stich and Laishes, 1973; Cleaver, 1974; Lieberman and Poirier, 1973,1974a,b) and tissues (Damjanov *et al.*, 1973; Lieberman and Forbes, 1973) undergo DNA repair synthesis in response to damage by ultraviolet radiation and by chemical mutagens (Fishbein *et al.*, 1970) and carcinogens (Irving, 1973). Relatively little is known in detail about the mechanism of repair in these cells. In particular, the process of nucleotide insertion following excision of damaged nucleotides has remained largely uncharacterized.

At the present time, analysis of the role of repair synthesis in such processes as mutagenesis and carcinogenesis in mammalian cells rests largely on data derived from investigation of repair in prokaryotic systems. Study of excision repair in prokaryotes suggests a model in which excision repair is error correcting (Kelly *et al.*, 1969; Kushner *et al.*, 1971); *i.e.*, that the base sequence on the undamaged complementary strand serves as a template for nucleotide insertion during repair synthesis. We wondered if we could find evidence for this model in intact mammalian cells. The importance of such a demonstration stems from the realization that, although elegant and sensitive techniques exist for assessing the fidelity of purified enzymes in copying well-defined polynucleotides (Kleppe *et al.*, 1971; Hershfield, 1973; Springgate and Loeb, 1973), it is not clear how well data from cell free systems reflect intracellular events (Burd and Wells, 1970; Loeb *et al.*, 1973). Furthermore, to date, there have been no definitive reports of purification of repair polymerases from mammalian sources.

To examine this problem, nondividing, confluent, human diploid fibroblasts (WI-38) and mouse Balb/c 3T3 cells were damaged with ultraviolet radiation (254 nm) or one of two mutagenic carcinogens [*N*-acetoxy-2-acetylaminofluorene[1] (Miller, 1970), NA-AAF; 7-bromomethylbenz[*a*]anthracene (Dipple *et al.*, 1971), 7BrMeBA] and labeled with [^{3}H]thymidine during subsequent DNA repair synthesis (Lieberman and Poirier, 1973,1974a,b). In order to examine how nucleotides are inserted after damage with these agents, we have used thermal elution chromatography on hydroxylapatite, S_1 nuclease digestion, and pyrimidine isostich profile analysis to evaluate base pairing and template specificity of DNAs labeled during repair synthesis on a comparative basis with DNAs labeled during replicative synthesis.

Materials and Methods

Cell Culture and Preparation of DNA. Preparation of DNAs labeled during repair synthesis has been described in detail elsewhere (Lieberman and Poirier, 1973,1974a,b). Briefly, WI-38 human diploid fibroblasts (passage 22-28) or mouse 3T3 cells were grown to confluence to obtain a resting, nondividing cell system. Any residual DNA synthesis from cells which escaped contact inhibition was suppressed by 10 mM hydroxyurea; 20 min later the cells were treated with ultraviolet radiation (200 erg/mm^2 = 20 J/m^2; 254 nm), *N*-acetoxy-2-acetylaminofluorene (10^{-5} M) (a gift of Dr. E. K. Weisburger), or 7-bromomethylbenz[*a*]anthracene (10^{-5} M) (a gift of Dr. A. Dipple); 20 min later (40 min after hydroxyurea) [^{3}H]methylthymidine (50 Ci/mmol, New Englad Nuclear) was added, and the cells were harvested 5 hr later. For experiments in which repaired DNA was to be isolated and characterized by physical techniques this system was used rather than one which employed asynchronous cultures and bromodeoxyuridine density labeling (see also Lieberman and Poirier, 1973,1974a). Since no foreign nucleotides (*e.g.*, bromouracil deoxyribonucleotide) are inserted during repair in this system, the physical properties of repaired DNAs can be compared directly to those of replicated DNAs without concern for possible alterations in the properties of repaired DNAs by substitution of varying amounts of bromodeoxyuridine for thymidine.

DNA was prepared directly in CsCl gradients from isolated nuclei (Lieberman and Poirier, 1973,1974b). When mouse DNA was to be fractionated into satellite and main-band DNA, DNA was prepared by phenol extraction and centrifugation in Ag^+–Cs_2SO_4 gradients (Lieberman, 1973;

† From the Experimental Pathology Branch, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20014. *Received July 22, 1974.*

‡ Present address: Environmental Mutagenesis Branch, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, North Carolina 27709.

[1] Abbreviations used are: 7BrMeBa,7-bromomethylbenz[*a*]anthracene; NA-AAF, *N*-acetoxy-2-acetylaminofluorene.

TABLE I: Summary of Hydroxylapatite Thermal Elution Profiles of Repaired and Replicated DNAs.

Type of Synthesis	Cell Type	$T_{E,50\%}$[b] (°C)	SD	No. of Determinations
Replicative	WI-38	87.4	±0.5	6
Repair after NA-AAF	WI-38	87.6	±0.8	5
Repair after 7BrMeBA	WI-38	87.4	±0.5	3
Repair after uvr[a]	WI-38	87.4		1
Replicative	3T3	86.9	±0.2	3
Repair after NA-AAF	3T3	86.5	±0.3	3
Repair after uvr	3T3	86.9	±0.4	2

[a] Uvr, ultraviolet radiation. [b] The temperature at which 50% of the radioactive DNA has been eluted from the column. The values were determined as described in the text and the legend to Figure 1.

Lieberman and Poirier, 1974a). Gradients used for preparation of DNAs labeled during repair synthesis were similar to those previously presented (Lieberman and Poirier, 1973, Figure 1; Lieberman and Poirier, 1974a, Figure 1; Lieberman and Poirier, 1974b, Figure 1). After treatment with ultraviolet radiation or chemical carcinogens there was a 15- to 60-fold increase in the specific activity of DNA from confluent cultures. In most cases, less than 5% of the label introduced under these conditions was the result of contamination by replicative synthesis (Lieberman and Poirier, 1974b). All DNAs were dialyzed into the appropriate buffer before use.

Labeled replicated DNAs were prepared from rapidly dividing asynchronous cells incubated for 6–12 hr with tritiated thymidine; DNA extraction was as described above, and DNAs from replicating cells and repairing cells were handled identically. Paper chromatography of nucleoside digests of both repaired and replicated DNAs indicated that 96–99% of the label was present as thymidine (Lieberman and Poirier, 1973).

Thermal Elution Chromatography. A detailed description of this method has been provided elsewhere (Lieberman and Poirier, 1974b).

S_1 Nuclease Digestion Studies. S_1 nuclease was purified through step 4 by the method of Vogt (1973); DNAs (about 10,000 cpm) in 0.2 ml of buffer A (Vogt, 1973) were mixed with 0.02 ml of enzyme preparation adjusted to contain about a tenfold excess of enzyme (*i.e.*, 10 times the amount needed to digest an equivalent amount of single-stranded DNA completely) and incubated at 36°. Single-stranded DNAs were prepared by heating to 100° for 4 min. Release into acid-soluble material was assayed by the addition of bovine plasma albumin carrier, precipitation with perchloric acid, and measurement of radioactivity in the supernatant after centrifugation.

Pyrimidine Isostich Analysis. Pyrimidine isostichs (runs) were prepared from [^{3}H]thymidine-labeled repaired and replicating mouse mainband and satellite DNAs. DNAs (2.5×10^4–2.5×10^5 cpm) were digested with formic acid diphenylamine (Černý *et al.*, 1968, 1969; Harbers and Spencer, 1974). After extraction of formic acid and diphenylamine with ether (Sneider, 1971), isostichs were separated on DEAE-cellulose columns (1 × 30 cm) with a 0.0–0.2 M NaCl linear gradient in 0.1 M sodium acetate buffer (pH 5.5) and 7 M urea (modified from the work of Spencer and collaborators by Lieberman *et al.*, 1971). Recovery of labeled nucleotides from columns was approximately 100%.

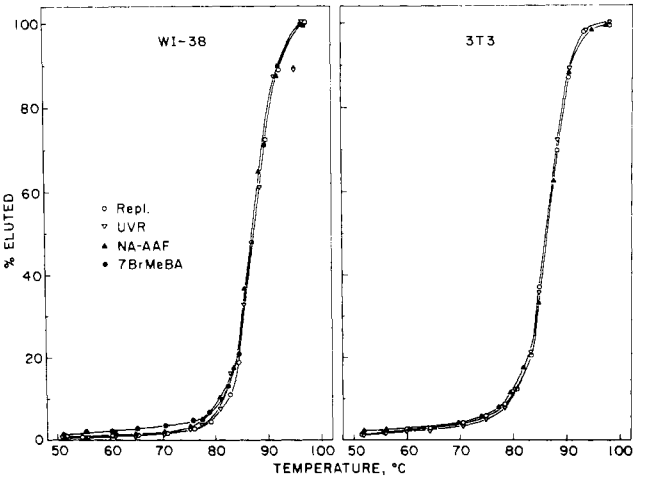

FIGURE 1: Thermal elution chromatography of DNAs labeled during repair or replicative synthesis. DNA samples labeled during repair or replicative synthesis were prepared as described under Materials and Methods. DNA was sonicated to 450 nucleotide length (Hoyer *et al.*, 1973), and 15,000–20,000 cpm (less than 75 μg; specific activity 200–20,000 cpm/μg) was loaded onto a hydroxylapatite column (2 ml packed volume, freshly boiled; Bio-Gel HTP, Richmond, Calif.) and eluted with a thermal gradient (Lieberman and Poirier, 1974b). Replicate samples eluted from different columns during the same run had $T_{E,50\%}$ values within 0.1–0.2°. Replicate samples sonicated and run on different days showed a range of 0.5–0.7°. Left panel: Thermal elution profiles of WI-38 DNA [(○) replicated DNA; (▽) DNA repaired after damage with uvr (200 erg/mm^2; 254 nm); (▲) DNA repaired after damage with NA-AAF (10^{-5} M); (●) DNA repaired after damage with 7BrMeBA (10^{-5} M)]. Right panel: Thermal elution profiles of 3T3 DNA (symbols are the same). The ordinate represents cumulative per cent of the total cpm eluted.

Results and Discussion

Thermal Elution Chromatography. DNAs labeled during repair and replicative synthesis were prepared (see Materials and Methods section) and subjected to thermal elution chromatography on hydroxylapatite (Lieberman and Poirier, 1974b). Thermal elution chromatography has been used to estimate the extent of sequence mismatch under a variety of conditions (McConaughy and McCarthy, 1970; Hough and Davidson, 1972; Crosa *et al.*, 1973; Grady and Campbell, 1973; Gummerson and Williamson, 1974). If the insertion of nucleotides during repair synthesis is template directed, then newly inserted nucleotides (identified by ^{3}H label) should be base paired with the complementary strand, and DNA sequences containing repair patches should have the same elution profiles and midpoint of elution ($T_{E,50\%}$) as those from replicated DNA. Inspection of

TABLE II: S_1 Nuclease Digestion of DNAs Labeled during Replicative or Repair Synthesis.

Type of Synthesis	Damaging Agent	Strandedness	Cell Type	Percentage Acid Soluble (10-min incubation)
Replicative		Native	3T3	2.8
Replicative		Native	3T3	2.3
Replicative		Native	WI-38	2.6
Replicative		Native	WI-38	0.8
Repair	Uvr	Native	3T3	2.6
Repair	Uvr	Native	WI-38	2.8
Repair	NA-AAF	Native	3T3	2.8
Repair	NA-AAF	Native	WI-38	3.7
Replicative		Denatured	3T3	97.3
Replicative		Denatured	WI-38	94.2
Repair	NA-AAF	Denatured	WI-38	100

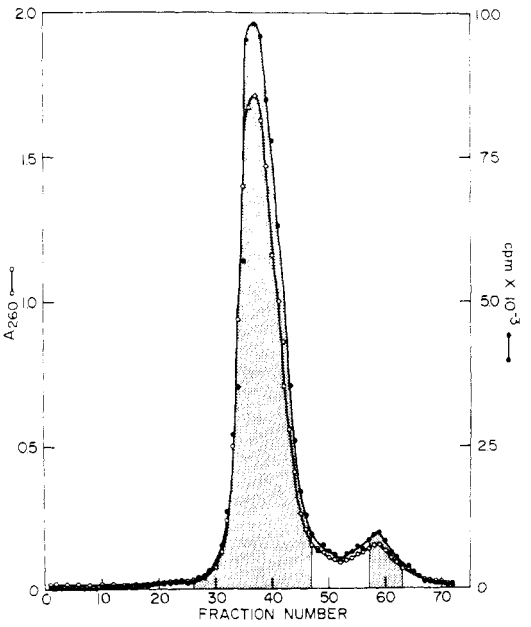

FIGURE 2: Separation of mouse DNA into mainband and satellite peaks. Mouse 3T3 cells were damaged with uvr and allowed to repair in the presence of [^{3}H]dT as described in the text; 500 μg of DNA was complexed with Ag^+ (molar concentration Ag^+/molar concentration DNA-P = 0.3) and centrifuged in a 10 ml Cs_2SO_4 gradient in a 50 Ti rotor (Beckman) as previously described (Lieberman and Poirier, 1974a). Similar gradients were run with DNA labeled during replicative synthesis. Left ordinate, A_{260} (O) of the diluted fraction; right ordinate, cpm $\times$ 10^{-3} (●) of a 0.1-ml aliquot. Fraction 1 represents the bottom of the gradient. Fractions 26–48 were pooled for mainband DNA while fractions 57–63 were used for satellite DNA.

profiles (Figure 1) and $T_{E,50\%}$ data (Table I) indicate that, within the limitations of the technique, DNAs labeled during repair synthesis or replicative synthesis have similar or identical elution profiles and $T_{E,50\%}$ values. The $-\Delta T_{E,50\%}$ (amount of lowering of the midpoint of the elution profile compared to a standard) may be used to estimate the extent of mismatch (Laird *et al.*, 1969; Ullman and McCarthy, 1973; Bonner *et al.*, 1973). In our studies the $-\Delta T_{E,50\%}$ ranges from 0.0 to 0.4° (Table I). Using the most conservative estimate of the relation between $-\Delta T_{E,50\%}$ and per cent mismatch (0.7°/1% mismatch) (Laird *et al.*, 1969) and knowing the piece size (450 nucleotides; legend to Figure 1) and the size of repair patches (100 nucleotides) (Cleaver, 1968; Regan *et al.*, 1971; Setlow and Regan, 1972; Setlow *et al.*, 1972), one may estimate that a 5% mismatch in the repair patch would result in at least a 1° lowering of the $T_{E,50\%}$ and would be detectable by this technique (Lieberman and Poirier, 1974b). These data suggest that the nucleotides inserted during repair synthesis are base paired with those on the complementary, undamaged strand.

S_1 Nuclease Digestion Studies. It was desirable to investigate base pairing in repair patches by an independent method. S_1 nuclease is an endonuclease with some exonucleolytic activity and a preference for single-stranded DNA. Thus regions of mismatch (single-stranded areas) may be detected as the release of acid-soluble nucleotides (Sutton, 1971; Crosa *et al.*, 1973; Vogt, 1973; B. H. Hoyer, personal communication). DNAs labeled during repair or replicative synthesis were digested under conditions designed to detect areas of mismatch (Table II). To reduce possible artifacts such as "sticking" or binding of enzyme to unremoved dimers or carcinogen or to trace RNA or protein contaminants, a tenfold excess of enzyme was used (see Materials and Methods section). It is apparent that a 10-min incubation at 36° has little effect on native (non-heat-denatured) DNAs whether labeled during repair synthesis or replicative synthesis. Incubation of samples for up to 1 hr produced only a small increase in the percentage of acid-soluble counts from nondenatured repair-labeled and replicative-labeled DNAs (data not shown). These findings are in agreement with the data from thermal elution chromatography studies: *i.e.*, DNA in repair patches behaves as double-stranded DNA.

Pyrimidine Isostich Profiles from Mouse Mainband and Satellite DNA. The template specificity of repair synthesis in intact cells may be examined by preparing pyrimidine isostich profiles (Černý, *et al.*, 1968, 1969; Lieberman *et al.*, 1971; Sneider, 1971) from mouse mainband and satellite DNA labeled during repair or replicative synthesis (Lieberman and Poirier, 1974a). The experiment takes advantage of the distinctively different isostich profiles of these two DNA species (Harbers and Spencer, 1974) (see also Figure 3). Thus, if repair synthesis is template-directed, isostich profiles from mouse satellite DNA labeled during repair synthesis should resemble those from satellite DNA labeled during replicative synthesis. The same is true, of course, for mainband DNAs. Mouse 3T3 cells were damaged with ultraviolet radiation (200 erg/mm^2; 254 nm) and DNA labeled during repair synthesis

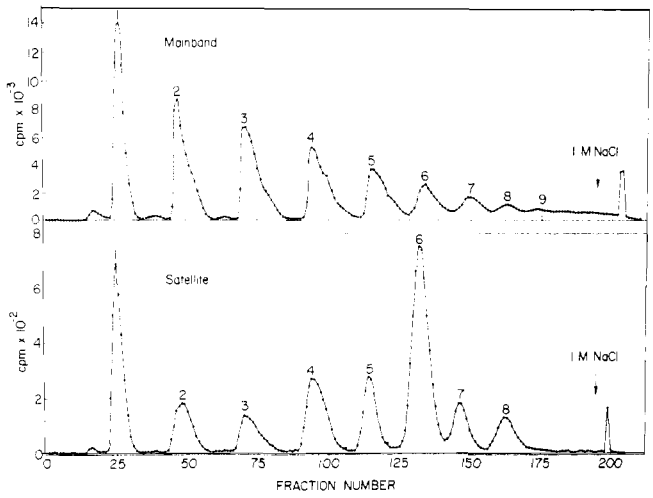

FIGURE 3. Pyrimidine isostich profiles of mouse mainband and satellite DNA labeled during repair synthesis after damage with ultraviolet radiation (see Materials and Methods section for details). Top panel: Isostich profile from mainband DNA labeled during repair synthesis. Bottom panel: Isostich profile from satellite DNA labeled during repair synthesis.

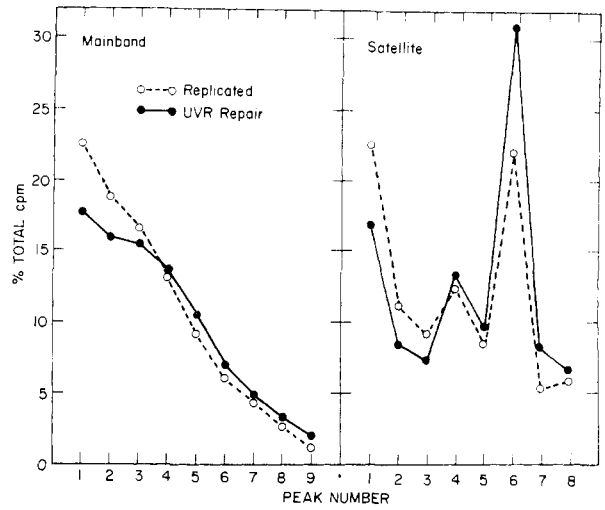

FIGURE 4: Distribution of [^{3}H]thymidine labeled nucleotides among isostichs from DNA labeled during repair synthesis after ultraviolet radiation or replicative synthesis. The percentage of total counts in each peak (ordinate) is plotted as a function of peak number (abscissa). DNA labeled during replicative synthesis (O - - - O); DNA labeled during repair synthesis (●—●). Left panel: Isostich profiles from mainband DNA. The symbols represent the average from two separate experiments; the size of the symbols approximates the range of values. Right panel: Isostich profiles from satellite DNA (single determination). There is a slight tendency for more ultraviolet-induced repair synthesis to occur in longer runs of pyrimidines, perhaps reflecting a tendency to form more thymidine-containing cyclobutyl dimers in long runs of pyrimidines (Brunk, 1973).

was separated into mainband and satellite DNA (Figure 2) (see also Lieberman and Poirier, 1974a). Similar separations were carried out on DNA labeled during replicative synthesis. After degradation of DNA with formic acid–diphenylamine, isostichs were separated by DEAE-cellulose chromatography (Figure 3), and the percentage of total counts eluted in each peak was plotted as a function of peak number (Figure 4). Clearly the profile from satellite DNA labeled during repair synthesis closely resembles that from satellite DNA labeled during replicative synthesis. Similar results are seen for the mainband DNAs. These data suggest that repair synthesis is template-directed and clearly rule out other models for repair synthesis such as random insertion, insertion of runs of a single nucleotide (*e.g.*, poly(dT), or insertion of runs of simple repeating units (*e.g.*, poly(dAT)). However, the method will not detect shuffled sequences (p-T-p-C-p-C-p-T *vs.* p-T-p-T-p-C-p-C-p) (Lieberman, *et al.*, 1971) or *occasional* substitutions of purines for pyrimidines (or *vice versa*) which would only slightly alter the relative abundance of various isostichs.

Implications for Mammalian Cells. The present studies suggest that repair synthesis in response to three mutagenic and carcinogenic agents in two different mammalian cell lines results in accurate restoration of the damaged strand to the original nucleotide sequence. It appears that gap-filling in association with excision is template-directed and that any errors introduced during the process are the result of the intrinsic error frequency of the polymerase itself in reading the template (Hershfield, 1973; Springgate and Loeb, 1973) and not of an error-prone, non-template-directed process. These data are in general agreement with prokaryotic models and imply that removal of potentially mutagenic sites (photoproducts and alkylated bases) before replication results in the conservation of genetic information. In contrast, post replication repair, according to a recent model (Lehmann, 1972), may restore linear continuity without conservation of information. The present studies demonstrate that repair synthesis in mammalian cells is not grossly inaccurate and represent a first step in evaluating the absolute template specificity (fidelity) of this process.

Acknowledgments

We thank Dr. Bill H. Hoyer, Dr. Nancy Reed Rice, and Dr. John H. Spencer for sharing their data with us prior to publication and Dr. C. Wesley Dingman, Dr. Valerio Genta, and Dr. David G. Kaufman for their thoughtful comments.

References

Bonner, T. I., Brenner, D. J., Neufeld, B. R., and Britten, R. J. (1973), *J. Mol. Biol. 81,* 123.

Brunk, C. F. (1973), *Nature (London), New Biol. 241,* 74.

Burd, J. F., and Wells, R. D. (1970), *J. Mol. Biol. 53,* 435.

Černý, R., Černa, E., and Spencer, J. H. (1969), *J. Mol. Biol. 46,* 145.

Černý, R., Mushynski, W. E., and Spencer, J. H. (1968), *Biochim. Biophys. Acta 169,* 439.

Cleaver, J. E. (1968), *Nature (London) 218,* 652.

Cleaver, J. E. (1974), *Advan. Radiat. Biol. 4,* 1.

Crosa, J. H., Brenner, D. J., and Falkow, S. (1973), *J. Bacteriol. 115,* 904.

Damjanov, I., Cox, R., Sarma, D. S. R., and Farber, E. (1973), *Cancer Res. 33,* 2122.

Dipple, A., Brookes, P., Mackintosh, D. S., and Rayman, M. P. (1971), *Biochemistry 10,* 4323.

Fishbein, L., Flamm, W. G., and Falk, A. L., (1970), Chemical Mutagens, Environmental Effects of Biological Systems, New York, N.Y., Academic Press.

Grady, L. J., and Campbell, W. P. (1973), *Nature (London), New Biol. 243,* 195.

Gummerson, K. S., and Williamson, R. (1974), *Nature (London) 247,* 265.

Harbers, K., and Spencer, J. H. (1974), *Biochemistry 13,* 1094.

Hershfield, M. S. (1973), *J. Biol. Chem. 248,* 1417.

Hough, B. R., and Davidson, E. H. (1972), *J. Mol. Biol. 70,* 491.
Hoyer, B. H., Rice, N. R., and Van de Velde, N. W. (1973), *Carnegie Inst. Wash. Yearb. 73,* 214.
Irving, C. C., (1973), *Methods Cancer Res. 7,* 189.
Kelly, R. B., Atkinson, M. R., Huberman, J. A., and Kornberg, A. (1969), *Nature (London) 244,* 495.
Kleppe, K., Ohtsuka, E., Kleppe, R., Molineux, I., and Khorana, H. G. (1971), *J. Mol. Biol. 56,* 341.
Kushner, S. R., Kaplan, J. C., Ono, H., and Grossman, L. (1971), *Biochemistry 10,* 3325.
Laird, C. D., McConaughy, B. L., and McCarthy, B. J. (1969), *Nature (London) 224,* 149.
Legator, M. S., and Flamm, W. G. (1973), *Annu. Rev. Biochem. 42,* 683.
Lehmann, A. R. (1972), *J. Mol. Biol. 66,* 319.
Lieberman, M. W. (1973), *Biochim. Biophys. Acta 324,* 309.
Lieberman, M. W., and Forbes, P. D. (1973), *Nature (London), New Biol. 241,* 199.
Lieberman, M. W., and Poirier, M. C. (1973), *Cancer Res. 33,* 2097.
Lieberman, M. W., and Poirier, M. C. (1974a), *Proc. Nat. Acad. Sci. U. S. 71,* 2461.
Lieberman, M. W., and Poirier, M. C. (1974b), *Biochemistry 13,* 3018.
Lieberman, M. W., Rutman, J. Z., and Farber, E. (1971), *Biochim. Biophys. Acta 247,* 497.
Loeb, L. A., Tartof, K. D., and Travaglini, E. C. (1973), *Nature (London) New Biol. 242,* 66.
McConaughy, B. L., and McCarthy, B. J. (1970), *Biochem. Genet. 4,* 425.
Miller, J. A. (1970), *Cancer Res. 30,* 559.
Regan, J. D., Setlow, R. B., and Ley, R. D. (1971), *Proc. Nat. Acad. Sci. U. S. 68,* 708.
Setlow, R. B., and Regan, J. D. (1972), *Biochem. Biophys. Res. Commun. 46,* 1019.
Setlow, R. B., Regan, J. D., and Carrier, W. L. (1972), *Biophys. Soc. Abs. 12,* 19a.
Sneider, T. W. (1971), *J. Biol. Chem. 246,* 4774.
Springgate, C. F., and Loeb, L. A. (1973), *Proc Nat. Acad. Sci. U. S. 70,* 245.
Stich, J. F., and Laishes, B. A. (1973), *Pathobiology Annu. 3,* 341.
Sutton, W. D. (1971), *Biochim. Biophys. Acta 240,* 522.
Ullman, J. S., and McCarthy, B. J. (1973), *Biochim. Biophys. Acta 294,* 416.
Vogt, V. M. (1973), *Eur. J. Biochem. 33,* 192.

Isolation of DNA Segments Containing Promoters from Bacteriophage T3 DNA†

T. Takeya and M. Takanami*

ABSTRACT: In order to isolate DNA segments containing promoters, bacteriophage T3 DNA was cleaved into about 60 unique fragments with endonuclease · Hap, which introduces duplex cleavages at the sequence of

---C/C-G-G---
---G-G-C/C---

(Sugisaki, H., and Takanami, M. (1973), *Nature (London), New Biol. 246,* 138). The specificity of RNA initiation was not influenced by this digestion, as far as the number of initiation and starting sequences for RNA synthesis was concerned. When the digest was incubated with either *Escherichia coli* RNA polymerase holoenzyme or T3 RNA polymerase, each enzyme was shown to form stable complexes with specific fragments depending on the combination of nucleoside triphosphates present in the binding mixture. The selectivity of binding by the host polymerase was lost if the σ factor was removed from the enzyme. Evidence that promoters are contained in the fragments isolated by the specific interaction with the polymerases was demonstrated with the fragment derived from the left end of T3 DNA. The procedures used in the present study should make possible isolation of DNA segments containing promoters from any DNA molecule.

Since the role of σ factor in the selection of initiation sites by RNA polymerase was elucidated (Burgess *et al.*, 1969; Krakow *et al.*, 1969; Travers and Burgess, 1969), a number of investigations have focused on the molecular mechanism of RNA initiation. However, little information is as yet available concerning the structure of RNA at which RNA synthesis is initiated. One of the difficulties in isolating the RNA initiation site (promoter) is that RNA polymerase can tightly bind to single-stranded regions of DNA, which are often created by the breakage of DNA (Hinkle *et al.*, 1972; Okamoto *et al.*, 1972). Recently, restriction endonucleases which degrade foreign DNA at a limited number of sites have been isolated from a variety of bacterial strains (for reviews, see: Boyer, 1971; Meselson *et al.*, 1972). As this type of enzyme introduces duplex cleavages on double-stranded DNA at a specific sequence of several nucleotides (Kelly and Smith, 1970, Hedgpeth *et al.*, 1972; Bigger *et al.*, 1973; Boyer *et al.*, 1973), these cleavages may not create nonspecific binding sites for RNA polymerase at the terminal regions. Accordingly, experiments were designed to isolate DNA segments containing promoters from bacteriophage T3 DNA after the cleavages with this type of enzyme. T3 DNA is known to provide at least two types of

† From the Institute for Chemical Research, Kyoto University, Uji, Kyoto-fu, Japan. *Received February 28, 1974.*