JOURNAL OF BACTERIOLOGY, Feb. 1975, p. 434-441

Vol. 121, No. 2
Printed in U.S.A.

Assay of Deoxyribonucleic Acid Homology Using A Single-Strand-Specific Nuclease at 75 C

PETER T. BARTH* AND NIGEL J. GRINTER

Department of Bacteriology, Royal Postgraduate Medical School, London W12 OHS, England

Received for publication 12 August 1974

We investigated the conditions under which a crude preparation of endonuclease S1 gives maximal hydrolysis of denatured deoxyribonucleic acid (DNA) while giving minimal hydrolysis of native DNA. The hydrolysis was measured by filtering and determining the acid-insoluble reaction product using ^{3}H-labeled substrates. We also investigated various parameters in making this measurement. Under appropriate conditions (in 1 mM $ZnSO_4$, 0.168 M NaCl at pH 4.8) denatured DNA is hydrolyzed within 3% of completion whereas native DNA is essentially unaffected. The reaction was applied to assay plasmid DNA homo- and heteroduplexes for which the method proves to be simple, fast, and reproducible.

Crosa, Brenner, and Falkow (5) have shown that the S1 endonuclease from *Aspergillus oryzae* (1, 10) can be used to assay deoxyribonucleic acid (DNA)-DNA hybridization of bacterial and plasmid DNA, and that the results, using a reassociation temperature of 60 C, closely match those obtained by the established hydroxyapatite method. The measurement of relative reassociation at 75 C by the two methods was more discrepant, however. It has been shown that the extent of base mismatching is less in DNA heteroduplexes formed at the higher temperature (3, 9) and, as expected, the hydroxyapatite method generally detected reduced reassociation at 75 C compared to 60 C. Crosa et al. found that the S1 endonuclease method detected no reduction at the higher temperature. This is presumably due to the fact that the S1 endonuclease reaction has generally been done at 50 C. During this reaction, which takes 20 min, there seems to be nothing to prevent heteroduplex strands that have been annealed at 75 C, for example, from increasing their strand pairing along distantly related sequences to the extent permitted by the 50 C temperature, thus increasing the apparent degree of homology. We are grateful to Nigel Godson, Yale University, for bringing this point to our attention. We have overcome this objection to the present method by demonstrating that S1 endonuclease works satisfactorily at the same temperature as the hybridization, up to at least 75 C, and that this reduces the measured degree of homology, especially for nonspecific, i.e., plasmid-chromosomal, DNA hybridizations.

The sensitivity of the S1 endonuclease reaction to chelating agents described by Crosa et al. has been diminished by increasing the $ZnSO_4$ concentration. The pH optimum and other conditions of the reaction have been investigated and altered, leading to an improved discrimination between denatured and native DNA by the nuclease.

MATERIALS AND METHODS

Preparation of S1 endonuclease. The enzyme was partially purified from the α-amylase of *Aspergillus oryzae* (Sigma Chemical Co.) according to Sutton's method (10). The first three 2-ml fractions of the final eluate from the diethylaminoethyl-cellulose column (Whatman DE-52) were found to contain sufficient single-stranded DNA nuclease activity in 5-μliter samples for use in the hybridization assay. These fractions were stored at −70 C in 25% (vol/vol) glycerol. The enzyme is stable for many months at −20 C, however.

Preparation of labeled DNA. [^{3}H]thymine-labeled plasmid DNA for hybridization was prepared from R$^+$ strains of *Escherichia coli* W3110T$^-$ by ethidium bromide CsCl density gradient isolation of covalently closed circular DNA molecules, as described previously (2). We used the chromosomal DNA band from such preparations as a substrate for the endonuclease assay. This was treated with isopropanol to remove ethidium bromide and dialyzed extensively against TES buffer [50 mM tris(hydroxymethyl)aminomethane, 5 mM ethylenediaminetetraacetic acid, 50 mM NaCl, pH 8.0] in the same way as the plasmid DNA. Samples were then diluted 100- to 400-fold with 0.42 M NaCl to give about 4×10^4 counts/min (180 ng of DNA) per ml. As a *thy*$^-$ strain was used for these ^{3}H-labelings, DNA concentrations

can be calculated from the counts per minute using the specific activity of the [^{3}H]thymine (333 μCi/μmol) and the efficiency of counting (37%).

Preparation of unlabeled DNA. Unlabeled plasmid DNA for use in hybridizations was prepared similarly to the sodium dodecyl sulfate (SDS)-NaCl method of Vapnek and Rupp (11) or of Guerry et al. (8). A 1.2-liter broth (plus 20 μM thymine) culture of the plasmid-containing strain W3110T$^-$ was grown overnight at 37 C with shaking. The cells were centrifuged, washed with TES buffer, and resuspended in 30 ml of ice-cold TES buffer containing 100 mg of sucrose and 1 mg of lysozyme per ml. This was kept in ice for about 10 min, when the cells were lysed by the addition of 7.5 ml of 5 M NaCl plus 1.5 ml of SDS (200 mg/ml). The lysate was stored at 4 C overnight and then centrifuged at 15,000 rpm (20,000 $\times$ *g*) for 30 min in a rotor (8 by 50 ml) of an MSE65 centrifuge. The clear supernatant solution was collected. In a scaled-down pilot experiment, using a ^{3}H-labeled culture of R300B/W3110T$^-$, we analyzed the DNA in the supernatant liquid at this stage by 5 to 20% sucrose gradient sedimentation analysis. We found approximately equal amounts of plasmid and chromosomal DNA. As the amount of plasmid DNA has been estimated to be about 2% of the chromosomal DNA in this strain (2), we conclude that the SDS-NaCl method has removed about 98% of the chromosomal DNA.

The DNA was further purified by treatment with an equal volume of TES-saturated phenol. The aqueous layer was collected and DNA was precipitated with 2 volumes of ethanol. This was centrifuged, and the pellet was dissolved in 10 ml of TES and treated with 50 μg of ribonuclease (Sigma Chemical Co.) per ml at 37 C for 45 min. The ribonuclease was heat-treated for 15 min at 85 C before use. After a second phenol extraction, the DNA solution was dialyzed at 4 C against four changes of 0.42 M NaCl over 48 h. DNA concentrations were measured by the Giles and Myers (7) modification of the Burton diphenylamine reaction.

DNA shearing and denaturation. DNA was sheared by ultrasonic treatment at 4 C with a Bronwill Biosonik II at 50-W energy output, in 30-s bursts with 30-s pauses for cooling, for a total of 5 min to an approximate molecular weight of 2.5 $\times$ 10^5 (6). It was denatured by heating for 10 min in a bath of polyethylene glycol (PEG 400, Shell Chemical Co.) at 101 C and either fast cooled in ice water or shifted to a 75 C water bath for renaturation as appropriate.

Preparation of plasmid DNA duplexes. ^{3}H-labeled plasmid DNA was diluted with 0.42 M NaCl to give about 10^4 counts/min per ml, sheared, and dialyzed overnight against two changes of 0.42 M NaCl. Samples (0.1 ml), containing about 4.5 ng of DNA, were mixed in silicone-treated tubes with 4 to 12 μg of sheared unlabeled plasmid DNA in a total volume of 0.4 ml, NaCl concentration being 0.42 M. This was denatured and then incubated at 75 C to a C_0t value (C_0 = initial DNA concentration, t = time) of about 10 $\times$ $C_0t_{1/2}$ (4). For plasmid DNA of molecular mass 5.7 Mdal, i.e., our linked streptomycin sulphonamide (SmSu) determinants (2), the incubation period was 90 to 150 min. $C_0t_{1/2}$ was measured empirically from the rate of homoduplex formation (see Fig. 4). $C_0t_{1/2}$ can also be calculated from Haapala's equation (D. K. Haapala, Ph.D. thesis, Georgetown University, Washington, D.C., 1969): $C_0t_{1/2}$ = molecular weight (M)/377 $\times$ 10^6. This empirical equation was derived for standard conditions of reassociation, viz., in 0.12 M phosphate buffer at 60 C using DNA fragments of molecular weight 125,000. Using more recent values for the weights of his tested DNA species (P22, 28 Mdal; T3, 25 Mdal; P1, 65 Mdal; T4, 120 Mdal; and R1, 60 Mdal) alters the denominator to 422 $\times$ 10^6. Reassociation in 0.42 M NaCl is quoted as being 3.4929 times faster than in 0.12 M phosphate buffer (13), thus $C_0t_{1/2}$ (0.42 M NaCl) = M/1,475 $\times$ 10^6. Converting the usual C_0t units (moles of nucleotides per liter $\times$ second) to the more practical micrograms of DNA/milliliter $\times$ min, i.e., multiplying by 332.5 $\times$ 10^3/60, gives $C_0t_{1/2}$ = M $\times$ 3.75 $\times$ 10^{-6}. The rate of reassociation of DNA fragments of 250,000 daltons at 75 C is not thought to differ much from 125,000-dalton fragments at 60 C; thus this final equation should apply reasonably well to our hybridization conditions.

S1 endonuclease reaction. The final method adopted for the endonuclease assay was as follows. At the end of the hybridization period, duplicate 0.4-ml hybridization mixtures were each supplemented with 0.6 ml of reaction mixture containing 0.1 ml of 0.3 M sodium acetate buffer (pH 4.8), 0.1 ml of 10 mM $ZnSO_4$, and 0.01 ml of sheared and denatured calf thymus DNA [2 mg of DNA/ml of 0.01 M tris(hydroxymethyl)aminomethane buffer, pH 7.5]. The reaction mixture was prewarmed to 75 C shortly before use. Duplicate single-stranded and double-stranded DNA controls of enzyme activity were also prepared from 0.1 ml of the ^{3}H-labeled DNA (as used in the hybridization mixture) plus 0.3 ml of 0.42 M NaCl or, where noted, mixtures identical to the hybridization mixture were used. The single-stranded DNA controls were denatured while the others were kept in ice. All the control tubes were then put in the 75 C bath and each was supplemented with prewarmed reaction mixture. Five microliters of S1 endonuclease was added to one of each pair of tubes immediately after addition of reaction mixture and the tube contents were mixed. The tubes were incubated at 75 C for a further 20 min and then cooled in ice water. One milliliter of ice-cold 10% trichloroacetic acid was added to each. The contents of each tube were filtered through a 25-mm diameter GF/C or GF/F filter (Whatman) which was washed with 4 $\times$ 2.5 ml of ice-cold 5% trichloroacetic acid containing 200 μM thymine and then with 2 ml of acetone. The filters were dried and put into vials containing 5 ml of scintillant (7 g of butyl-2-(4$^+$-t-butylphenyl)-5-(4''-biphenyl)-1,3,4-oxadiazole/liter of toluene), and the radioactivity was measured in an Intertechnique ABAC SL40 liquid scintillation spectrometer.

Calculation. The ratios of counts from each pair of the three pairs of tubes used in a hybridization give: s, the proportion of single-stranded DNA not hydrolyzed by the enzyme; d, the proportion of double-stranded DNA not hydrolyzed by the enzyme; h, the proportion of DNA after hybridization not hydrolyzd by the enzyme. If H is the proportion of DNA hybridized, then h = Hd + (1 - H)s and H = (h - s)/(d - s).

RESULTS

Effect of temperature. The hydrolysis of sheared heat-denatured and native ^{3}H-labeled chromosomal DNA by our partially purified S1 endonuclease preparation at various temperatures is shown in Table 1. Although it has been previously reported that the enzyme is inactivated at 60 to 65 C (1), our results demonstrate that, over a 20-min time course at least, temperatures up to 75 C can be used without any detriment. Measurement of the reaction kinetics at 75 C shows that the hydrolysis of denatured DNA is essentially complete after 8 to 10 min.

Effect of zinc ion concentration. S1 endonuclease was reported by Ando (1) to be optimally stimulated by zinc ions (0.1 mM). $ZnSO_4$ concentrations varying from 0.03 mM (10) to 1 mM (12) have been used in the reaction. We have tested this effect under our conditions at 75 C. The results are shown in Fig. 1, where it can be seen that the single-stranded nuclease activity of the enzyme is inhibited by $ZnSO_4$ concentrations above 2 mM. We tested the 0.1 to 1.0 mM $ZnSO_4$ concentration range for the sensitivity of the reaction to small amounts of contaminating material (ethylenediaminetetraacetic acid or SDS) in the DNA substrate. This sensitivity was reported by Crosa et al. (5). The results in Table 2 demonstrate that, for such substrates, increasing the $ZnSO_4$ concentration to 1.0 mM reduces the percentage of unhydrolyzed denatured DNA from unacceptably high to low levels. We have therefore adopted 1.0 mM $ZnSO_4$ for our standard nuclease reaction conditions, despite the slightly greater hydrolysis of native DNA at this concentration seen in Fig. 1 and Table 2. This hydrolysis was reduced by adjusting the reaction pH (next section).

TABLE 1. *Action of S1 endonuclease on single-stranded and double-stranded DNA at various temperatures*[a]

Temp (C)	Trichloroacetic acid-precipitable material (%)	
	Denatured DNA substrate	Native DNA substrate
50	5.4	89
60	6.1	86
70	5.3	82
75	6.6	89

[a] Duplicate 100-μliter samples of sheared ^{3}H-labeled chromosomal DNA (as described), either native or denatured, were incubated for 20 min at the given temperature with 0.9 ml of prewarmed reaction mixture containing 0.3 ml of 0.42 M NaCl, 0.1 ml of 0.3 M sodium acetate buffer (pH 4.5), 0.1 ml of 10 mM $ZnSO_4$, and 0.01 ml of sheared denatured calf thymus DNA [2 mg of DNA per ml of 0.01 M tris(hydroxymethyl)aminomethane buffer, pH 7.5], and the addition of 5 μliters of enzyme to one of each pair of tubes. Cold 5% trichloroacetic acid-precipitable material was collected and counted as described, except that HA filters (Millipore Corp.) were used and the acetone wash was omitted.

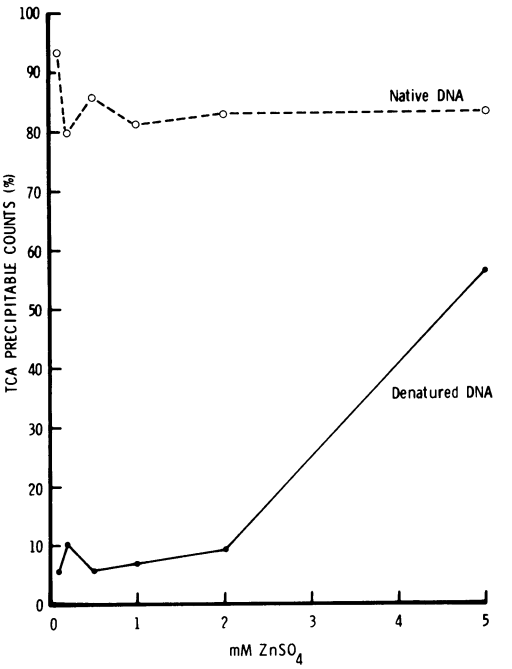

FIG. 1. *Effect of $ZnSO_4$ concentration. The S1 endonuclease reaction was carried out at 75 C, with various $ZnSO_4$ concentrations, as described in Table 1. GF/F filters and the washing procedure given in Materials and Methods were used.*

$ZnSO_4$ (1 mM) reduces, but does not eliminate, differences between the residual unhydrolyzed, denatured DNA of different DNA substrates. This variation (3 to 10%) makes it necessary to measure this residuum for each labeled DNA preparation used in a hybridization.

pH optimum. Ando (1) has reported a pH optimum of 4.5 for the single-stranded endonuclease reaction at 37 C and Vogt (12) 4.0 to 4.3 at 45 C. Different reaction conditions and purity of enzyme preparation were used. For the purposes of a hybridization assay, we sought to optimize not only the maximal hydrolysis of single-stranded DNA, but also the minimal hydrolysis of double-stranded DNA. Figure 2 shows the activity of the nuclease on both substrates at 75 C in 1.0 mM $ZnSO_4$. It can be seen that optimal conditions are met with over a

TABLE 2. *Effect of $ZnSO_4$ concentration on the sensitivity of the nuclease reaction to contaminating material in the DNA substrate*[a]

DNA substrate	Trichloroacetic acid-precipitable material (%)	
	0.1 mM $ZnSO_4$	1.0 mM $ZnSO_4$
Denatured (no. 8)	33.3	3.9
Native (no. 8)	85.7	83.0
Denatured (no. 9)	21.2	4.6
Native (no. 9)	86.7	80.3

[a] Two ^{3}H-labeled chromosomal DNA preparations that had not been extensively dialyzed (no. 8 and no. 9) were used as substrates of the endonuclease reaction in the presence of 0.1 and 1.0 mM $ZnSO_4$. Reaction conditions were as described under Fig. 1.

rather narrow pH range, viz. 4.7 to 4.9. We have adopted pH 4.8 for our standard conditions. The change from the previously used pH 4.5 (5, 10) slightly improves the hydrolysis of denatured DNA, but is especially important, under our conditions, in reducing the hydrolysis of native DNA: the unhydrolyzed percentage of 83% increases to essentially 100% (see Table 4).

Effect of NaCl concentration. S1 endonuclease has been shown to be influenced by salt concentration, Sutton (10) reporting optimal activity on denatured DNA in 0.1 M NaCl. Crosa et al. (5) noted a reduction in hydrolysis of native DNA between 0.15 to 0.2 M NaCl without a significant reduction in hydrolysis of denatured DNA. Under our reaction conditions, the results are similar (Fig. 3). The curves show a broad plateau between 0.1 to 0.25 M NaCl with 0.2 M being close to optimum.

Figure 3 also demonstrates the outcome achieved by our final method. The reproducibility is good: most duplicate points are less than ±1% from their mean. At 0.168 M NaCl, which we have used for hybridization assays, less than 3% of the denatured DNA and apparently more than 100% of the native DNA are left unhydrolyzed. The latter anomaly is dealt with in the next section.

Carrier effect of the enzyme. The 110% recovery of native DNA after addition of the enzyme, compared to the no-enzyme parallel reaction, is also demonstrated in Table 3. The addition of heat-inactivated enzyme (100 C for 20 min), or of native enzyme for zero reaction time, does not effect a similar recovery of native DNA (Table 3). Neither does the addition of 100 μg of calf thymus DNA (unsheared) or 100 μg of bovine serum albumin to the reaction mixture (1 ml) after the reaction, before filtration, increase the recovery of denatured or native DNA (data not shown). The carrier effect thus requires native S1 endonuclease and time (the reaction period). It is, therefore, presumably due to the enzyme binding to short DNA fragments in the sheared native DNA mixture and preventing their subsequent solubilization by 5% trichloroacetic acid. Without removing such presumed fragments, it is difficult to see how to avoid the anomaly. For calculation purposes, we normalize the native DNA hydrolysis to this 110% level.

Effect of filter type. We have tested various types of filters for the collection of trichloroacetic acid-insoluble material from the nuclease reaction, both membrane and glass fiber types. Table 4 shows their comparative retention after the reaction of both denatured and native DNA substrates. Apart from the Oxoid and Sartorius 11307 membrane filters which retain too little native DNA after reaction, all the filters are satisfactory. We use glass fiber in preference to membrane filters (Millipore Corp.) because (i) they cost less than a tenth as much and (ii) the efficiency of isotope counting is higher by 22 to 25% (the quench is less as shown by the ratio of counts in channel A [restricted ^{3}H window for

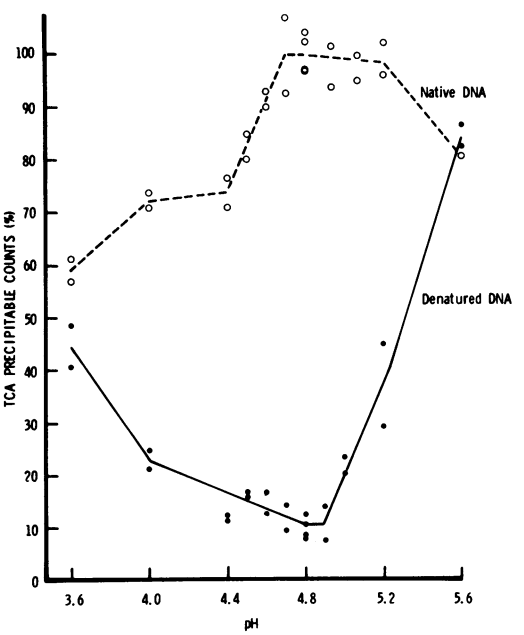

FIG. 2. *pH dependence of nuclease activity on denatured and native DNA. The S1 endonuclease reaction was carried out at 75 C in 1.0 mM $ZnSO_4$ and 30 mM sodium acetate buffers of pH 3.6 to 5.6. The other conditions were as in Fig. 1. The results are of two experiments, the first covering the whole range in 0.4 pH units and the second covering pH 4.5 to 5.0 in 0.1 pH units.*

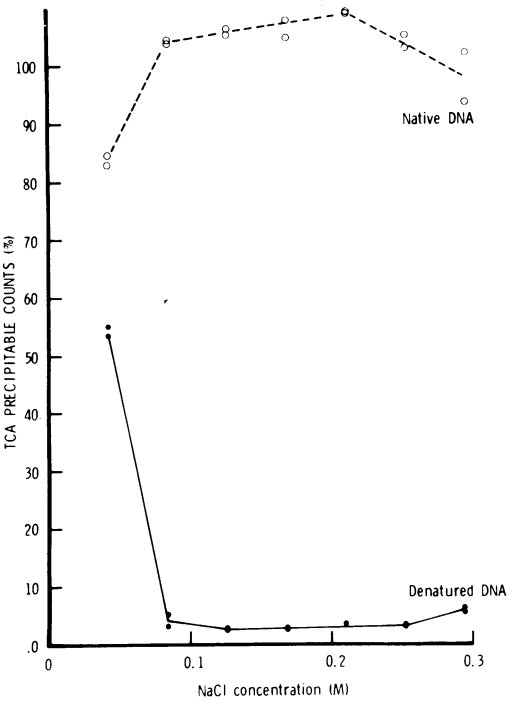

FIG. 3. *Effect of NaCl concentration. The hydrolysis of denatured and native ^{3}H-labeled chromosomal DNA by S1 endonuclease at 75 C in 1.0 mM $ZnSO_4$ at pH 4.8 was measured in the presence of various NaCl concentrations (in 0.042 M steps) by the usual procedure. GF/F filters were used. All the DNA samples that were denatured were in 0.42 M NaCl during the 100 C period.*

^{3}H plus ^{14}C counting] to channel C [full ^{3}H window] being 0.869 for GF/Fs and 0.942 for HA filters [Millipore] in our scintillation counter). It is our impression that GF/F filters (pore diameter 0.7 μm) give slightly better reproducibility than GF/C filters (pore diameter 1.2 μm).

Other reaction conditions. We use silicone-treated tubes for hybridizations and nuclease reactions as we have found that they enhance reproducibility. We have not further investigated the anomalous reaction kinetics described by Sutton (10), viz. that low concentrations of denatured DNA are incompletely hydrolyzed. As suggested by him, this is overcome by the addition of 20 μg of sheared denatured (calf thymus) DNA/ml to the reaction mixture. We find that the trichloroacetic acid concentration used for DNA precipitation and washing is not critical: over the range 2 to 6.5% there was no significant change in recovery of counts.

Kinetics of homoduplex formation. We tested the S1 endonuclease method and measured the $C_0t_{½}$ value of our unlabeled plasmid (R300B) DNA preparations by measuring the rate of homoduplex formation between the latter and a ^{3}H-labeled R300B preparation. Several identical hybridization mixtures were set up as described above, denatured at 100 C, and then incubated for various periods at 75 C before the addition of prewarmed reaction mixture and enzyme. Trichloroacetic acid-precipitable material was collected and counted as usual. The results are presented in Fig. 4 in the form C_0/C versus time, where C_0 is the initial DNA concentration and C is the single-stranded DNA concentration. DNA-DNA reassociation has been shown to follow second order kinetics (13), i.e., $(C_0 - C)/C_0 = Ckt$ (k = rate constant, t = time). Thus, $C_0/C = 1 + k C_0 t$ and therefore C_0/C is proportional to t. (C_0/C is the reciprocal

TABLE 3. *Carrier effect of the native S1 endonuclease on native DNA*[a]

Enzyme added	Trichloroacetic acid-precipitable material (%)	
	Denatured DNA substrate	Native DNA substrate
None	100	100
Native	2.9	110.8
Heat-inactivated	94.8	88
Native (zero time reaction)	100.8	101.3

[a] Reactions were performed in triplicate under our standard conditions (Materials and Methods) with native enzyme, heat-inactivated enzyme (100 C for 20 min), or with native enzyme for as short a time as practically possible. The recovered radioactive counts are expressed as a percentage of the no-enzyme reaction.

TABLE 4. *Comparison of various types of filters in the retention of nuclease reaction products*[a]

Filter type	Trichloroacetic acid-precipitable material (%)	
	Denatured DNA substrate	Native DNA substrate
Millipore (GS)	9.7	88.0
Millipore (HA)	6.6	86.6
Oxoid Nuflow	5.9	70.6
Sartorius 11307	11.7	79.2
Whatman GF/F	6.8	89.4
Millipore (HA)	6.6	100.3
Whatman GF/F	8.5	101.0
Whatman GF/C	8.8	102.8

[a] Duplicate reactions were performed at 75 C as previously described: the first five rows at pH 4.5, the last three at pH 4.8. Filtration and washing procedure was as described, but the acetone wash was omitted from the membrane filters.

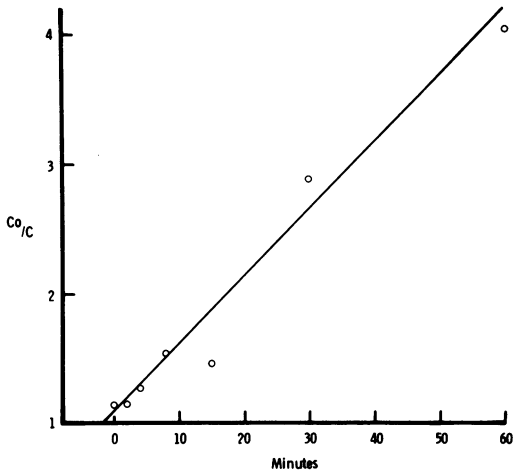

FIG. 4. *The kinetics of R300B DNA reassociation. Samples (0.1 ml) were removed at the times shown from a hybridization mixture containing 45 ng of ^{3}H-labeled R300B DNA per ml plus 13 μg of unlabeled R300B DNA per ml in 0.42 M NaCl which was denatured and incubated at 75 C. The samples were supplemented with 0.9 ml of reaction mixture and enzyme and were incubated as described. Denatured and native DNA controls were also set up. Trichloroacetic acid-precipitable material and the proportion of DNA reassociated were measured as in Materials and Methods. C_0/C is the reciprocal of the proportion of DNA remaining unassociated.*

of the proportion of DNA in single-stranded form, i.e., 1/1 − H). The slope of such a plot is kC_0 which is $1/t_{½}$, where $t_{½}$ is the time for 50% reassociation (i.e., $C_0/C = 2 = 1 + kC_0t_{½}$).

The results in Fig. 4 lend credance to the validity of the method as the measured rate of reassociation fits the expected second order kinetics reasonably well. From the slope, $t_{½}$ = 19.4 min. As C_0 was 13 μg/ml, $C_0t_{½}$ = 252 μg/ml × min. Repeat measurements with other R300B DNA preparations gave similar $C_0t_{½}$ values (242 to 263). Assuming our plasmid DNA concentration is 50% of the unlabeled DNA preparations, as indicated by the pilot measurement, then $C_0t_{½}$ for R300B is 126 μg/ml × min. This differs considerably from the equation developed from Haapala's data above, i.e., $C_0t_{½}$ = 5.7 × 3.75 = 21.4 μg/ml × min. The reason for this discrepancy is not known. We have used our measured $C_0t_{½}$ values to establish the C_0t conditions for heteroduplex formation between R300B and the isolated SmSu plasmid DNA samples (2).

Comparison of 50 and 75 C assays. It has been argued above that assaying heteroduplexes at a lower temperature than that at which they were annealed will lead to reassociation of mismatched base pairs and therefore give an apparently higher degree of homology. We have tested this directly by hybridizing DNA from three I group plasmids and two bacterial genera, *Proteus mirabilis* and *E. coli* at 75 C, and then assaying them at 50 C according to the Crosa et al. (5) method and at 75 C by our method. The results in Table 5 show that the 75 C assay conditions lead to somewhat improved discrimination between denatured and native DNA by the enzyme (although this was less complete using the plasmid DNA samples than using chromosomal DNA as reported in preceding sections). No significant difference is found for the homoduplex assays (R483 and *E. coli* K-12) at the two temperatures. This is expected as all the nucleotide sequences are fully matched. For the related I plasmid (S. Falkow, P. Guerry, R. W. Hedges, and N. Datta, J. Gen. Microbiol., in press) heteroduplex assays, however, the 50 C assay gave significantly higher values than the 75 C assay. Also, the nonspecific, plasmid-chromosomal DNA values were significant at 50 C but nicely insignificant at 75 C.

DISCUSSION

Crosa et al. (5) have developed a simple, reproducible, and quick method for the assay of DNA-DNA duplexes, dependent on the use at 50 C of the single-strand-specific endonuclease S1 from *Aspergillus oryzae*, and have shown that results obtained by this method correlate well with those obtained by the hydroxyapatite method when assaying 60 C hybridizations, but less well with 75 C hybridizations. We have therefore investigated the conditions involved in the use of this enzyme at 75 C. The optimal conditions established result in essentially no hydrolysis of native DNA and hydrolysis of single-stranded DNA within 3% of completion. This is an improvement over the previous method, but, more important, permits the assay of 75 C hybridizations without any temperature shift and concomitant alteration of the degree of DNA strand pairing. We have shown that a temperature shift to 50 C leads to a significantly increased estimate of strand pairing in related plasmid DNA heteroduplexes formed at 75 C.

We have also reduced the sensitivity of the reaction to contaminating chelating agents, etc., in the DNA. Caution should be used in applying our conditions to temperatures other than 75 C. The pH optimum we find has a narrow range and differs from previous findings at lower temperatures (1, 12). The various conditions we have adopted are also, presumably, interdependent.

TABLE 5. *Comparison of 50 and 75 C S1 endonuclease assays of various 75 C DNA-DNA reassociations*[a]

DNA mixture		Trichloroacetic acid-precipitable material (%)					
		Denatured DNA control		Native DNA control		Hybridization	
^{3}H-labeled DNA	Unlabeled DNA	50 C	75 C	50 C	75 C	50 C	75 C
R483	R483/J62	14.5	9.0	108.7	109.6	79.2	74.8
R144	R483/J62	29.8	13.0	91.3	105.4	65.0	37.2
JR66a	R483/J62	17.7	14.5	105.9	103.6	80.2	40.3
R483	*E. coli* J62	12.9	9.9	97.8	100.3	28.4	1.9
R144	*E. coli* J62	12.4	12.2	95.6	96.7	32.3	0.7
JR66a	*E. coli* J62	16.2	12.3	101.7	108.9	36.8	3.3
P. mirabilis 13	*E. coli* J62	3.2	3.6	93.0	91.1	4.6	0.0
E. coli W3110T$^-$	*E. coli* J62	7.7	2.1	98.5	95.3	103.7	95.3
Averages		14.3	9.6	99.0	101.4		

[a] Sheared ^{3}H-labeled DNA samples (0.1 ml) were mixed with 0.3 ml of sheared unlabeled DNA isolated from *E. coli* J62 or R483/J62. (As total DNA was isolated, the spheroplasts were lysed with sarkosyl [8 μg/ml] instead of SDS and NaCl, and the DNA was purified as described.) The unlabeled DNA concentration was 150 μg/ml in the mixtures. After denaturation and hybridization for 22 h at 75 C, mixtures were assayed in duplicate for reassociated DNA using the S1 endonuclease method at 75 C as described above, and at 50 C as described by Crosa et al. (5). The control mixtures (identical to the hybridization mixtures) were either denatured or left as native DNA and were similarly assayed by both methods. The hybridization results are presented without normalization to the homologous reaction nor subtraction of the nonspecific (plasmid-chromosomal) reaction.

It will be noticed that, rather than using unlabeled whole bacterial DNA containing 2 to 5% plasmid DNA, we have generally utilized partially purified (SDS-NaCl lysis) plasmid DNA for hybridizations. In nonspecific hybridizations with 100 to 700 μg of chromosomal DNA/ml, R300B gave 4 to 8% duplex formation. This becomes negligible when the chromosomal DNA concentration is only a few micrograms per milliliter in the experimental hybridization, and enables us to omit a nonspecific hybridization of each plasmid DNA.

Advantages and disadvantages of an enzyme method compared to hydroxyapatite or bound DNA methods have been discussed (5). Theoretically an enzyme method should give a more satisfactory assay of DNA-DNA heteroduplexes than either of the other techniques. Whereas the latter are thought not to recognize a short single-stranded loop in a long reassociated DNA segment, a single-strand-specific endonuclease can hydrolyze such a loop (although it is not known how small a loop can be recognized by endonuclease S1). The reverse situation of a long single-stranded piece of DNA containing a small double-stranded region should also be better resolved by a nuclease method.

The method has much to recommend it. The enzyme is cheap and easy to prepare in large quantities and remains stable at −20 C over several months with repeated thawings and freezings. The technique requires no specialized apparatus, can deal with large numbers of assays at a time, and, with reasonable care, gives rapid and reproducible results.

ACKNOWLEDGMENTS

We are indebted to S. Falkow and his colleagues at the University of Washington, Seattle, for their hospitality to one of us (PTB) in June 1973 to learn their DNA hybridization techniques.

We are grateful to the Medical Research Council of the U.K. for support for this visit, and for their support of this research by a grant to N. Datta.

LITERATURE CITED

1. Ando, T. 1966. A nuclease specific for heat-denatured DNA isolated from a product of *Aspergillus oryzae*. Biochim. Biophys. Acta **114**:158–168.
2. Barth, P. T., and N. J. Grinter. 1974. Comparison of the DNA molecular weights and homologies of plasmids conferring linked resistance to streptomycin and sulphonamides. J. Bacteriol. **120**:618–630.
3. Brenner, D. J., and S. Falkow. 1971. Molecular relationships among members of the *Enterobacteriaceae*. Advan. Genet. **16**:81–118.
4. Britten, R. J., and D. E. Kohne. 1966. Nucleotide sequence repetition in DNA. Carnegie Inst. Wash. Year Book **65**:78–106.
5. Crosa, J. H., D. J. Brenner, and S. Falkow. 1973. Use of a single-strand specific nuclease for analysis of bacterial and plasmid deoxyribonucleic acid homo- and heteroduplexes. J. Bacteriol. **115**:904–911.
6. Falkow, S., and R. V. Citarella. 1965. Molecular homology of F-merogenote DNA. J. Mol. Biol. **12**:138–151.
7. Giles, K. W., and A. Myers. 1965. An improved diphenylamine method for the estimation of deoxyribonucleic acid. Nature (London) **206**:93.
8. Guerry, P., D. J. LeBlanc, and S. Falkow. 1973. General

method for the isolation of plasmid deoxyribonucleic acid. J. Bacteriol. **116**:1064–1066.
9. Johnson, J. L., and E. L. Ordal. 1968. Deoxyribonucleic acid homology in bacterial taxonomy: effect of incubation temperature on reaction specificity. J. Bacteriol. **95**:893–900.
10. Sutton, W. D. 1971. A crude nuclease preparation suitable for use in DNA reassociation experiments. Biochim. Biophys. Acta **240**:522–531.
11. Vapnek, D., and W. D. Rupp. 1971. Identification of individual sex-factor DNA strands and their replication during conjugation in thermosensitive DNA mutants of *Escherichia coli*. J. Mol. Biol. **60**:413–424.
12. Vogt, V. M. 1973. Purification and further properties of single-strand-specific nuclease from *Aspergillus oryzae*. Eur. J. Biochem. **33**:192–200.
13. Wetmur, J. G., and N. Davidson. 1968. Kinetics of renaturation of DNA. J. Mol. Biol. **31**:349–370.