DNA-DNA HYBRIDIZATION AT LOW TEMPERATURE USING DNA CHEMICALLY LABELED WITH ^{14}C-DIMETHYL SULFATE[1]

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SUMMARY

Purified P22 and P221 DNAs were labeled by methylation with ^{14}C-dimethyl sulfate at 4°C. This labeled moiety was unstable at high temperature. About 60% of the radioactivity was released from the DNA by incubation at 65°C for 20 hrs. Using the ^{14}C-methylated DNA, the maximal efficiency of DNA-DNA hybridization was obtained by hybridization at 27°C in the presence of 30% formamide. Determination of the homology between partially genetically related phages proved that this hybridization technique is a useful tool for genetic study.

INTRODUCTION

The routine method for obtaining radioactively labeled DNA is to culture organisms in the presence of radioactive precursors. However, some organisms and viruses are extremely difficult to culture, require rich nutrients or are relatively impermeable to radioactive precursors because of their cell membrane or wall. Thus, it becomes extremely difficult to incorporate radioactive precursors into DNA. Moreover in DNAs of some animals, calf thymus DNA for example, it is impossible to obtain radioactively labeled nucleic acids by feeding radioactive precursors. However, these nucleic acids can be chemically labeled by incubation with alkylating agents.

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Using DNA methylated with ^{14}C-dimethyl sulfate, we attempted DNA-DNA hybridization at 65°C and obtained an insignificantly low isotopic activity. Similar results were also observed by Searey (1968). The low activity with this hybridization technique could be due to depurination of methylated guanine.

In this communication, we report *in vitro* methylation of DNA by incubation with ^{14}C-dimethyl sulfate at 4°C and hybridization of ^{14}C-methylated DNA with unlabeled DNA under various conditions.

MATERIALS AND METHODS

Salmonella bacteriophages P22c2 and P221bc2 which were grown on *Salmonella typhimurium* Q1 were used. P22 and P221 are serologically unrelated yet they carry a large homologous region (Yamamoto, 1969). 1XSSC contained 0.15M NaCl and 0.015M Na-citrate (pH 7.3). Standard preincubation medium (PM) contained 0.02% of Ficoll, ploy vinylpyrrolidone, and bovine serum albumin in 3 x SSC (Denhardt, 1966). ^{14}C-Dimethyl sulfate (specific activity, 3.2 mc/mM) was purchased from New England Nuclear Corp.

P22 and P221 particles were purified by repeated differential centrifugation and by CsCl centrifugation. Phage DNAs were extracted with water saturated phenol and precipitated by addition of 2 volume of cold ethanol. The precipated DNA was washed, freed of ethanol and suspended in distilled water.

The phage DNA (500 µg) was mixed with 100 µc ^{14}C-dimethyl sulfate (DMS) in 0.4 ml of 0.1 M Na-citrate buffer at pH 7.5. This reaction mixture was incubated at 4°C. After a 48 hr incubation, the mixture was treated with 0.5 M NaOH for 60 min. followed by neutralization with HCl. This alkaline treatment denatures DNA and also stabilizes methylated bases (Brookes and Lawley 1960). The DNA was precipitated by addition of 3 volumes of ethanol. The precipitated DNA was further washed 6 times to remove free DMS molecules and resuspended in 1 x SSC adjusted at pH 8.0. In some instances, Sephadex G-100 was used instead of the alcohol precipitation procedure. The recovery and the specific radioactivity (average 1,500 cpm/µg) of labeled DNAs in both cases were the same. From the specific activity, one nucleotide per about 100 nucleotides was methy-

lated. This DNA preparation was sonicated for 1 min by Branson Sonifier Model S-125 (Branson Instrument Inc. Danbury Conn.). More than 95% radioactivity of this sample was acid precipitable after 1 month storage at -20°C.

The purified unlabeled phage DNA was denatured by heat treatment in a 1 x SSC solution. The denatured phage DNAs were diluted to 5 ml with 6 x SSC and passed through a millipore membrane filter HA (presoaked in 2 x SSC for overnight), then washed with about 20 ml of 6 x SSC. The DNA filters were subsequently dried in a vacuum desicator overnight and further incubated at 60°C for 3 hrs. The dried filters were preincubated in PM solution in scintillation vials at 60°C for 1 hour then cooled down to the room temperature. The ^{14}C-methylated DNA was added to the vials and annealing was carried out under various conditions. The filters were then removed from the hybridization fluid and each side was washed with about 100 ml of 2 x SSC. Finally, the filters were dried and counted in a Packard Tricarb Scintillation counter.

RESULTS

The purified ^{14}C-methylated DNA was incubated at various temperatures in

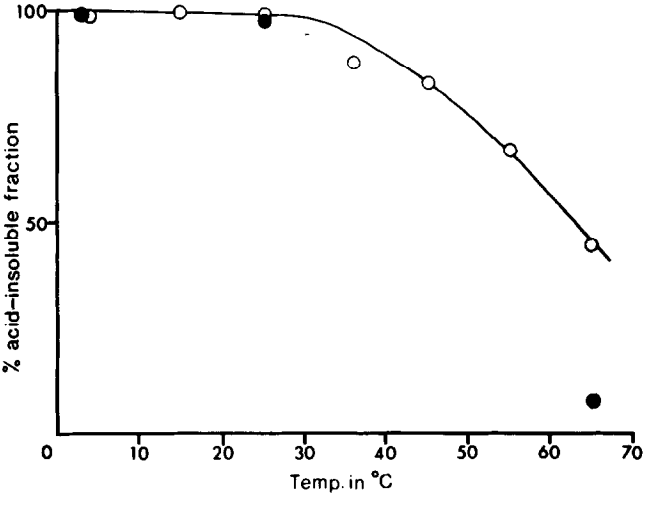

Fig. 1. Heat stability of the ^{14}C-methylated DNA. Symbols: Alkaline treated DNA (O), untreated DNA (●).

2 x SSC. After 20 hr incubation, DNA was precipitated by 5% cold trichloroacetic acid, collected on millipore filter, washed, dried and counted. As shown in Fig. 1, at high temperature the labeled moiety was unstable.

When hybridization was performed at various temperatures, the maximal hybridization was obtained at 45°C (Fig. 2). However, since depurination of methylated guanine occurs at this temperature, these conditions may result in inconsistency of hybridization data. Thus, it is desirable to use lower temperature for hybridization.

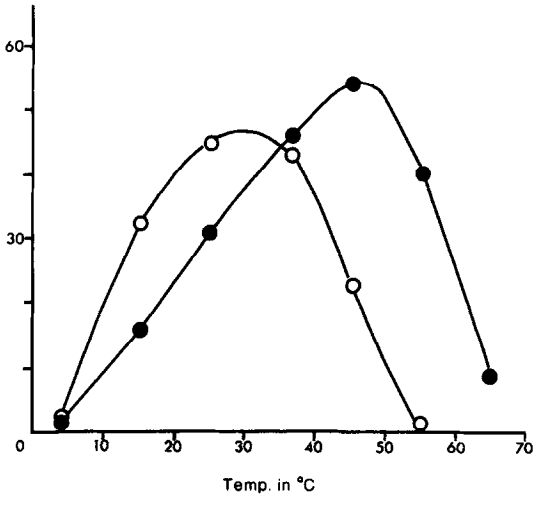

Fig. 2. Effect of temperature on the hybridization. The filters holding 50μg unlabeled P22 DNA was incubated with 7.6μg P22 DNA (6,000 cpm) at various temperature for 25hrs with (O) or without (●) the presence of 30% formamide.

Bonner et al. (1967) reported that addition of 30% formamide results in an increased efficiency of DNA-RNA hybridization at 25°C. Thus we have tested effect of formamide on efficiency of DNA-DNA hybridization. When various concentrations of formamide were studied, as shown in Fig. 3, 30% formamide gave the maximal efficiency of DNA-DNA hybridization at 25°C. Moreover, when 30% formamide was used, the maximal hybridization was obtained

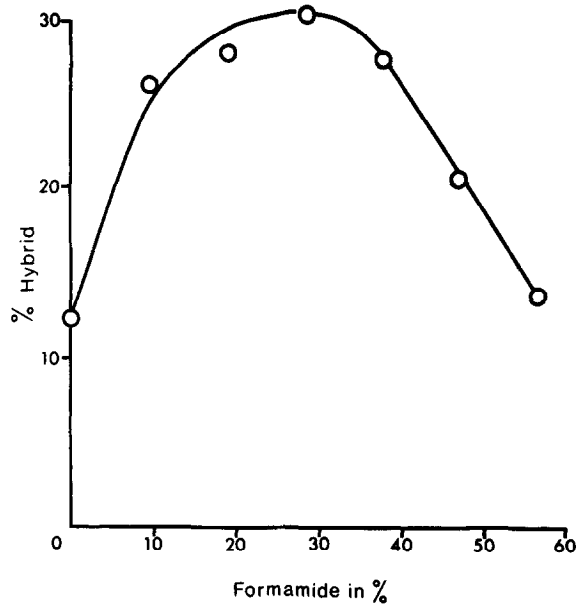

Fig. 3. Effect of formamide concentration on the DNA-DNA hybridization. The filter holding 20μg P22 DNA was incubated with 7.6μg P22 DNA (6,000 cpm) in 2XSSC with the presence of various concentration of formamide (vol. %) at 27°C for 25 hrs.

at about 27°C (Fig. 2). Under these conditions, hybridization increased as incubation period prolonged and reached the maximum value at 25 hrs.

When the filters holding various amounts of unlabeled P22 and P221 DNAs were incubated in a constant amount of the ^{14}C-methylated P22 DNA solution in 30% formamide at 27°C for 25 hrs, the values for hybridization with both P22 and P221 DNAs increased as amounts of cold DNAs increased as shown in Fig. 4a

Counts for hybridization of the ^{14}C-methylated P22 DNA with P22 DNA were higher than those with P221 DNA throughout DNA concentrations tested. Thus, it is evident that the value for the homologous phage DNA is higher than that for the partially related phage DNA. These values should give the ratio of the length of the homologous region to the entire P22 genome. Using this technique, it was found that the physical length of the homologous region is about 30% of the P22 genome. Moreover, when the ^{14}C-methylated P221 DNA was

hybridized with unlabeled P221 DNA and P22 DNA, it was found that the physical length of the homology is about 40% of P221 genome (Fig. 4b). The discrepancy of these values suggests that the P221 genome is smaller than the P22 genome and agrees with our separate study by sucrose gradient centrifugation (Akiyoshi and Yamamoto, in preparation).

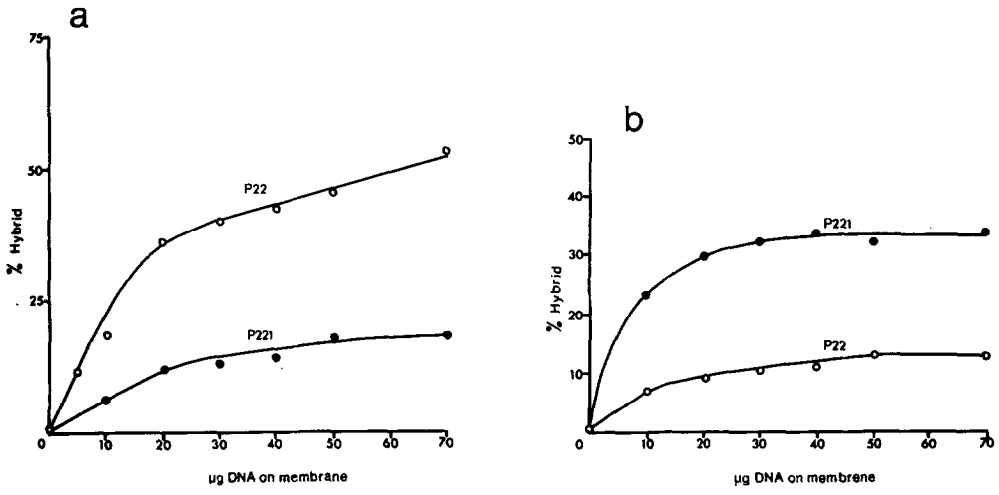

Fig. 4. Hybridization between genetically related bacteriophages P22 and P221. A constant amount (7.6µg; 6,000 cpm) of the ^{14}C-methylated P22 DNA (a) or a constant amount (1.5µg; 2,100 cpm) of the ^{14}C-methylated P221 DNA (b) was hybridized with various amounts of P22 and P221DNAs at 27°C for 25 hrs in 2XSSC with the presence of 30% formamide.

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