STUDIES ON HYBRID MOLECULES OF NUCLEIC ACIDS
I. DNA-DNA HYBRIDS ON NITROCELLULOSE FILTERS

J.Legault-Démare, B.Desseaux, T.Heyman, S.Séror and G.P.Ress
Institut du Radium, Radiobiologie, 91 Orsay, France

Received July 10, 1967

The technique of Gillespie and Spiegelman (1965) for the detection of DNA-RNA hybrids is based on the observation that nitrocellulose is able to adsorb denatured DNA, although RNA and native DNA are not retained (Nygaard and Hall, 1964).

The same principle may be applied to the study of DNA-DNA homology if only one of the two DNA's to be compared is immobilized. This requirement is fulfilled in the technique of Denhardt (1966) which makes use of a mixture of high polymers to prevent the adsorption of the DNA to be tested on the excess free sites of the nitrocellulose filters carrying the reference DNA. In the technique of Warnaar and Cohen (1966), hybridization is carried out as for DNA-RNA hybrids, and the non-hybridized DNA is subsequently eluted, leaving the hybrid molecules adsorbed on the membrane.

In this paper, we describe a technique of formation of DNA-DNA hybrids on membranes, based upon the observation that in the presence of moderate amounts of dimethyl sulfoxide (DMSO), denatured DNA is not retained by the nitrocellulose, although it is still able to renature or to hybridize with homologous DNA.

Materials and Methods

Membranes : Membranfilter Gesellschaft, Göttingen, Germany, type MF 50, diameter 25 mm.

Dimethyl sulfoxide : purissimum Fluka, Switzerland.

DNA was extracted from T2 phage by the phenol technique of Mandell and Hershey (1960). ^{3}H and ^{32}P labels were introduced in the form of thymidine and of ortho phosphate respectively during phage growth.

Fixation of DNA on membranes : ^{3}H labeled, heat denatured T2 DNA in 6xSSC was slowly filtered through the membranes, which were subsequently washed and dried according to Gillespie et al. (1965). The amount of DNA retained on the filters was usually 85 to 90% of the input, and the experiments described in this paper were done with membranes loaded with about 20 µg of DNA (10^4 counts/min.under our counting conditions).

Melting curves : The denaturation-renaturation profiles were measured in a Beckman DU Spectrophotometer equipped with dual thermospacers. A special lid permitted the introduction of a thermoelectric couple inside a reference cuvette in the cuvette holder, for the accurate measure of temperature.

After heating well above the point of complete denaturation, the renaturation curves were obtained during slow cooling of the circulating fluid (100 to 30°C in 3 hours). The concentration of DNA in these experiments was about 15 µg/ml ($O.D._{260}$ = 0.300), and the concentration of DMSO varied from 0 to 40% (v/v).

Counting of radioactivity: The dried membranes were placed in scintillation vials and counted in a Packard TriCarb spectrometer ; the cross-contamination of ^{32}P in the ^{3}H canal was of 0.66%.

Results

1 - Fixation of DNA on membranes in the presence of DMSO.

The membranes were washed with 50 ml of 2xSSC or 6xSSC containing 0 to 40% DMSO (v/v). 40 µg of labeled denatured T2 DNA in 5 ml of the same mixture were slowly filtered through the washed membranes. The filters were finally washed with 100 ml of the same mixture, dried, and the radioactivity was determined.

Fig.1 shows that in the presence of 20% DMSO, the fixation of the

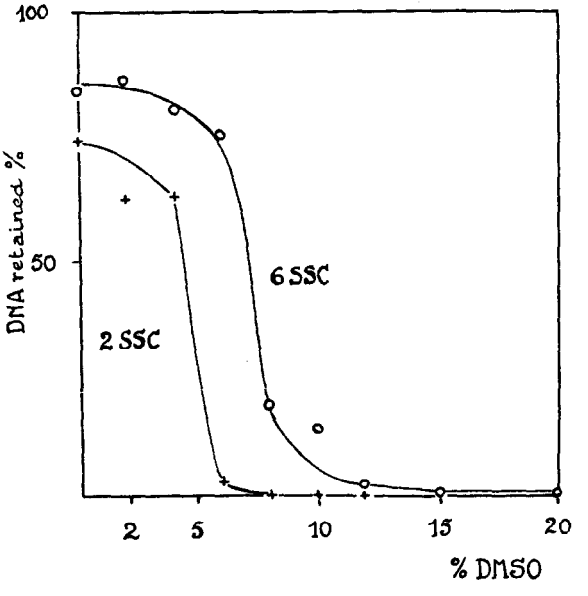

Fig 1 . Filtration of denatured T2 DNA through nitrocellulose filters in the presence of different concentrations of DMSO . See details in the text.

DNA to the filters was almost completely abolished. However, a small amount of radioactivity (about 0.4%) was retained, even in the presence of 40% DMSO. This contamination was fairly constant, and was not modified when the DNA was submitted to repeated pronase and phenol treatments ; however, it was reduced when the solution of denatured DNA in 2xSSC-30% DMSO was filtered several times through nitrocellulose membranes. The significance of this phenomenon has not been investigated.

2 - Denaturation and renaturation of DNA in the presence of DMSO.

A number of substances are known to lower the T_M of DNA (Helmkamp and Ts'o, 1961 ; Marmur and Ts'o, 1961 ; Hanlon, 1966).

Fig.2 presents two of the denaturation-renaturation profiles obtained in the experimental conditions described under "Methods". It can be seen that in the presence of 30% DMSO, the T_M of T2 DNA was in fact lowered by 14°C, but that the renaturation curve was displaced by the same value. Although in these conditions complete renaturation was not achieved, reheating of the renatured DNA showed that the renatured portion had the same T_M than native T2 DNA.

These results allowed us to conclude that hybridization as well

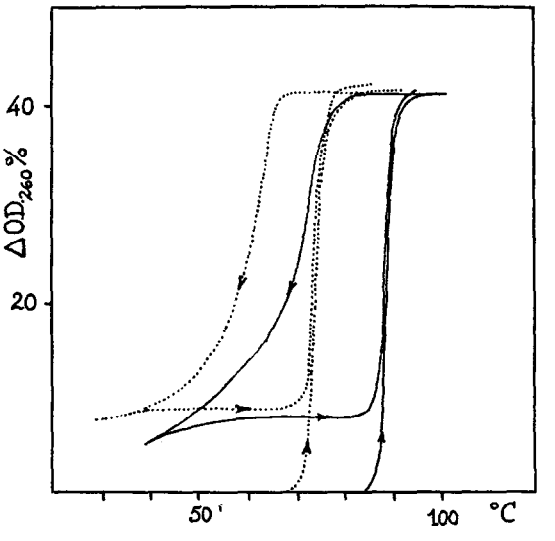

Fig 2 Denaturation-renaturation profiles of T2 DNA. Concentration : 15 µg/ml, in 2xSSC (full line) or in 2xSSC containing 30% DMSO (dotted line).

as renaturation should be possible in the presence of DMSO.

3 - DNA-DNA hybridization.

The following experimental conditions were found to give the best results in studying DNA-DNA hybridization ; the subscript letters refer to the comments.

Technique. The membranes, loaded with ^{3}H labeled reference DNA(a) following Gillespie et al (1965), and the same number of blank filters, were soaked for a few minutes in cold 2xSSC containing 30% DMSO (v/v)(b).

Each labeled membrane was transferred with one blank filter into a small plastic vial (diameter 30 mm) containing 1 to 5 µg (c) of denatured ^{32}P input DNA in 4ml of 2xSSC-30% DMSO(d).

The vials were gently agitated in a thermostatic bath during the desired time(e).

After incubation, the vials were cooled in ice, and the membranes were washed on a Büchner funnel, on one side with 50 ml of 2xSSC containing 30% DMSO, on the other side with 100 ml of 2xSSC.

The membranes were dried under an infra-red lamp, and the radioactivity was determined.

Comments.

(a) The term"reference DNA"applies to the DNA fixed to the membranes, and "input DNA"to the DNA introduced into the hybridization medium.

(b) Dry filters introduced directly into the reaction mixture yielded higher blanks.

(c) The main factor to be controlled is the concentration of denatured input DNA in the solution. The best results were obtained with 0.5 to 2.0 µg/ml. Higher concentrations gave higher blanks, owing to the adsorption to the filters of a small percentage of the input DNA.

(d) Although 10 to 15% DMSO were enough to eliminate the adsorption of DNA on the membranes during a filtration, it was necessary to use 20 to 30% of this compound to prevent blank contamination during incubation.

On the other hand, this amount of DMSO in 2xSSC did not alter the filters even at 60°C, and did not remove the DNA which had been previously fixed. The figures listed in column 2, table 1, show that for a given batch of loaded membranes, the amount of reference DNA remaining on the filters after incubation lay in the range of 85 to 90% of the controls, except in 1xSSC, 30% DMSO, 60°C, where it was of only 70%.

Owing to the variations observed between simultaneously prepared filters, it was considered necessary to use labelled reference DNA to secure a

reasonable precision in the results of the experiments.

(e) The yield and the % hybrid were significantly enhanced when the vials were gently shaken during the incubation : in Table I, samples II-11 and 12 which were shaken, gave results 1.8 times higher than samples II-9 and 10 which were not.

Expression of the results : The ratio $\frac{\mu g \text{ input DNA hybridized}}{\mu g \text{ DNA on the filter}} \times 100$ is designated by % hybrid, and the yield of the reaction is defined by the ratio $\frac{\mu g \text{ input DNA hybridized}}{\mu g \text{ total input DNA}} \times 100$.

Results : This technique was tested with the system T2 ^{3}H DNA (reference DNA) against T2 ^{32}P DNA (input DNA). Table I and figs 3 to 5 show the results obatined in different experiments. The optimum temperature was found to be 40 to 50°C in 2xSSC, 30% DMSO (fig 3). In fig 5, the results of a competition experiment are presented, showing a normal competition between cold T2 DNA and ^{32}P T2 DNA, and no competition between cold thymus DNA and ^{32}P T2 DNA.

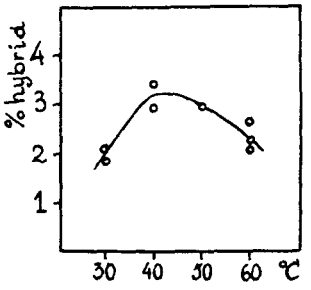

Fig 3 . Variation of the extent of hybridization in function of temperature. Membranes carrying an average of 18 µg of ^{3}H T2 DNA were incubated for 16 hours in 2xSSC, 30% DMSO at the indicated temperatures, in the presence of 2 µg of ^{32}P T2 DNA.

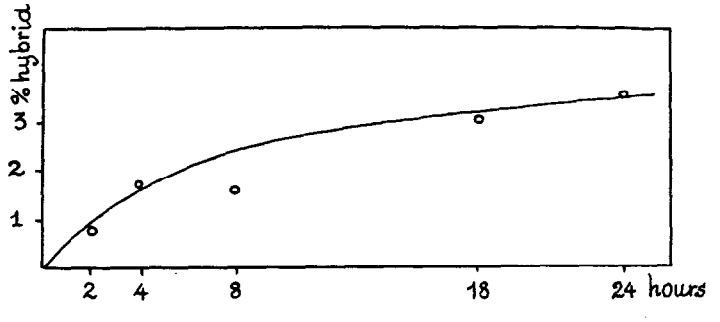

Fig 4 . Kinetics of DNA-DNA hybridization. Conditions as in fig 3 ; the temperature was maintained at 50° for the desired time.

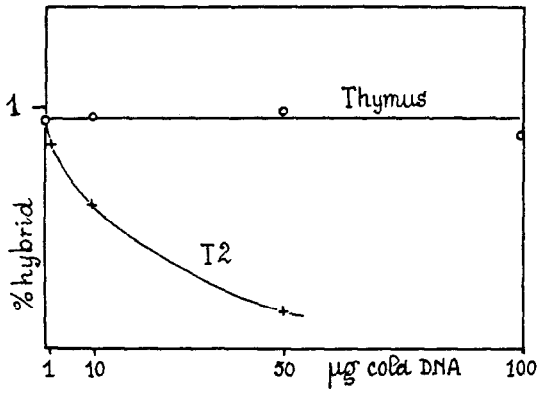

Fig 5 . Competition experiment. Membranes carrying an average of 20 µg of ^{3}H T2 DNA were incubated for 16 hours at 50°C in 3ml of 2xSSC, 30% DMSO, containing 1 µg of ^{32}P T2 DNA and increasing amounts of denatured cold competitor DNA.

N°	DNA on the filter µg	inputDNA µg/ml	solvent volume ml.SSC	solvent DMSO %	temperat. °C	hybrid %	yield	blank % of input
I- 1	27.7	2.0	5 x 2	20	60	3.1	8.6	0.23
2	25.8	2.0	5 x 2	20	60	1.9	4.9	0.65
II- 1	15.8	0.33	3 x 1	30	30	0.45	6.8	0.30
2	16.6	"	"	"	"	0.46	7.6	
3	16.5	"	"	"	40	0.38	6.2	
4	16.3	"	"	"	"	0.40	6.5	
5	16.3	"	"	"	50	0.48	7.8	
6	14.3	"	"	"	"	0.50	7.3	
7	11.0	"	"	"	60	0.70	7.7	
8	11.5	"	"	"	"	0.64	7.2	
9	15.9	0.5	4 x 2	30	50	2.3	18.	0.22
10	15.0	"	"	"	"	2.1	16.	0.25
11	17.3	"	"	"	"	3.2	27.	0.33
12	15.4	"	"	"	"	4.0	31.	0.27
III- 1	18.1	0.5	4 x 2	30	30	2.13	19.3	0.30
2	18.4	"	"	"	"	1.30	16.5	0.30
3	15.6	"	"	"	40	3.45	27.	0.17
4	16.3	"	"	"	"	2.90	24.	0.18
5	20.4	"	"	"	50	3.0	30.	0.32
6	18.7	"	"	"	60	2.06	19.3	0.11
7	15.8	"	"	"	"	2.21	17.5	0.18
8	17.0	"	"	"	"	2.74	23.2	0.23

Table I : Hybridization ^{3}H T2 DNA x ^{32}P T2 DNA in different conditions . solvent : 5 x 2 means 5ml of 2xSSC. Incubation time for all experiments : 16h. Samples from II- 9 to III- 8 were shaken during the incubation. Input DNA in sample I- 2 was submitted to shearing in a VirTis homogeneizer, 15min at

10.000 rpm at 0°. I, II and III correspond to three batches of loaded filters. Control filters were counted either directly or after incubation in 2xSSC 16 hours at 60°. The following values were obtained :
Batch II : 17.3, 14.8, 17.7, 15.5 µg ; mean value : 16.4 ± 0.8. (incubated)
Batch III : 23.0, 19.6 (incubated) ; 17.6,22.6 (not incubated).

Discussion

The technique presented here permits hybridization of analogous DNA's at relatively low temperatures, in the presence of a denaturing agent. This technique is analogous to the procedure of Denhardt (1966), but eliminates the necessity of a pretreatment of the membranes.

In accordance with the properties of denatured DNA and of nitro cellulose, Denhardt found 80% of the input DNA bound to blank filters if the pretreatment was omitted. Thus, in the technique of Warnaar et al (1966), as no preincubation is used, the same amount of input DNA must be lost by adsorption to the filters. The remaining 20% input DNA may then yield renatured molecules, either in the solution, or with the adsorbed 80% ; it may also eventually form hybrid molecules with the reference DNA. Apparently, this technique takes advantage of the drying procedure of Gillespie et al which results in a very strong linkage between nitrocellulose and denatured reference DNA. Consequently, the small percentage of input DNA actually hybridized is not removed by the washing procedure at low ionic strength, which eliminates the input DNA directly attached to the filters.

In the procedure presented here, as in the technique of Denhardt, the input DNA can only either renature or hybridize with reference DNA ; however, the yields obtained by the three techniques are of the same order of magnitude. In addition, competitive hybridizations between homologous DNA's were recently obtained (Hotta et al., 1966), although the input DNA adsorbed on the filters was not eliminated at all, even by the unexpected RNAse treatment proposed by the authors.

All these data lead to the conclusion that the so-called DNA-DNA hybridization techniques on membranes are based on one of the multiple reactions which may occur reversibly when free denatured DNA, adsorbed denatured DNA and excess nitrocellulose are annealed together and submitted to various empirical treatments. One must be very careful in interpreting the results of such experiments.

References

D.T.Denhardt, Biochem.Biophys.Res.Commun.,23(1966)641.

D.Gillespie and S.Spiegelman, J.Mol.Biol.,12(1965)829.

S.Hanlon, Biochem.Biophys.Res.Commun., 23(1966)861.

G.K.Helmkamp and P.O.P.Ts'o, J.Amer.Chem.Soc., 83(1961)138.

Y.Hotta, M.Ito and H.Stern, Proc.Natl.Acad.Sci.,56(1966)1184.

J.D.Mandell and A.D.Hershey, Anal.Biochem., 1(1960)66.

J.Marmur and P.O.P.Ts'o, Biochim.Biophys.Acta, 51(1961)32.

A.P.Nygaard and B.D.Hall, J.Mol.Biol., 9(1964)125.

S.O.Warnaar and J.A.Cohen, Biochem.Biophys.Res.Commun., 24(1966)554.