IMPROVED CONDITIONS FOR IN SITU RNA/DNA HYBRIDIZATION

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1. Introduction

The aim of the present investigation was the development of a method for *in situ* RNA/DNA hybridization in which the hybridization reaction can be carried out at a low temperature, over long periods with small volumes of RNA solutions. Fulfillment of this aim would facilitate the localization of sequences which are not highly repetitive and of sequences with melting points well below that of the major portion of the nuclear DNA of eukaryotes.

Thus far, the hybridization reaction in *in situ* hybridization experiments have been performed at an incubation temperature of 65° with labeled RNA dissolved in 2 X SSC [1] or 6 X SSC [2]. However, it was indicated that under these conditions reassociation of the chromosomal DNA may occur within a short period of time [3–5]. This process, favoured by the proximity of complementary DNA strands in the chromosomes, should reduce the efficiency of the RNA/DNA hybrid formation considerably, and in effect bias the hybrid formation in the direction of most highly repetitive sequences.

The present report describes a method for *in situ* hybridization in the presence of formamide at a temperature of 20°. Formamide was used during hybridization previously by Jones and Robertson [6]. However, they report that under their conditions it was less satisfactory than 65° in 2 X SSC.

2. Materials and methods

2.1. *RNA used for in situ hybridization*

Complementary RNA (cRNA) was prepared by *in vitro* transcription of 8 μg nuclear DNA obtained from mid-third instar salivary gland nuclei of *Drosophila hydei*. The DNA was transcribed with 5 units of *E. coli* RNA-polymerase (Sigma) in the presence of 50 μCi of each of the four tritiated nucleoside triphosphates [7]. The RNA was extracted from the transcription medium according to Perry et al. [8], and had a specific activity of 7×10^7 dpm/μg.

In vivo synthesized RNA was obtained from *Drosophila hydei* larvae grown for 7 days on a medium containing 1 mCi/ml [^{3}H]uridine and was extracted as described previously [9]. The RNA had a specific activity of 2×10^6 dpm/μg.

From the *in vivo* synthesized RNA, 18 S and 28 S RNA fractions were separated by acrylamide gel electrophoresis [10]. The pooled 18 S + 28 S fractions had a specific activity of 2.1×10^6 dpm/μg.

The purified RNA was dissolved in 1 X SSC and, by the addition of SSC and/or formamide, made to a final concentration of 6 SSC or 50% formamide and 2 X SSC before applying it to the cytological preparations.

2.2. *Cytological preparations used for in situ hybridization*

Mid-third instar larval salivary glands of *Drosophila hydei* were dissected, fixed for 10 min in ethanol–acetic acid (3:1, v/v), squashed in 45% acetic acid and, after removal of the coverslip, postfixed for 15 min in the previously used fixative, then transferred to 70% and subsequently to 100% ethanol. After rinsing in 2 X SSC the preparations were incubated for 60 min with pancreatic RNAase (100 μg/ml) in 2 X SSC, rapidly chilled and extensively rinsed with 2 X SSC [11, 12].

Denaturation was performed by a 2.5 hr incubation of the preparations in 90% formamide in

Table 1
Radioactivity in [^{3}H]cRNA solutions recovered after *in situ* hybridization to slides which were previously submitted to sham hybridization for various periods.

A) Sham hybridization at 65° in 6 × SSC followed by hybridization with [^{3}H]cRNA (152,928 cpm/slide) in 50% formamide, 2 × SSC at 20° for 60 min.

Period of sham hybridization (min)	Radioactivity in cpm recovered per slide*	Radioactivity in cpm retained per slide*
none	125,428	27,500
10	138,030	14,898
60	147,426	5,502
120	148,485	4,443
180	147,021	5,907

B) Sham hybridization at 20° in 50% formamide, 2 × SSC followed by hybridization as in A.

none	124,628	28,300
10	127,190	25,738
60	129,457	23,471
120	129,828	23,100
180	131,227	21,701

* Average of two slides per series.

0.1 × SSC at 65° [13] followed by immediate transfer into ice cold 0.1 × SSC, subsequently to ice cold 70% and 100% ethanol and finally drying under vacuum at low temperature.

The use of a coverslip to cover the RNA solution after its application to the cytological preparation was avoided in experiments carried out at a low temperature in order to enable an almost quantitative recovery of the volume applied. The incubation of the preparation with the RNA solution was performed in a sealed plastic petri dish containing filter paper wetted with the solvent of the RNA applied.

Following the incubation, the solution was removed, pooled with 4 washes of 50 μl 2 × SSC and the recovered radioactivity determined by scintillation counting in 15 ml of a mixture of toluene:ethoxy–ethoxy ethanol (6:4, v/v). The slides were extensively rinsed and treated with 20 μg/ml RNAase for 180 min at 25°, rinsed again and subsequently coated with Kodak AR 10 stripping film [14]. The slides were exposed for 6 days in those experiments in which cRNA was applied and for 2 months in the experiments using RNA synthesized *in vivo*.

It should be pointed out that the denaturation procedure, the processing of the slides, and photographic procedures were identical for all slides. Moreover, the preparations were made of salivary glands from larvae of essentially the same developmental stage. Each preparation contained only one gland which normally contains about 130 polytene nuclei [15], or about 0.06 μg DNA.

3. Results and discussion

In order to compare the relative effectiveness of the hybridization reactions performed at 65° or in the presence of formamide at 20°, two series of slides, in which each slide contains essentially the same amount of DNA, were denatured and each series was submitted to different hybridization conditions in the *absence* of RNA. Following application of these conditions for various periods of time (table 1), the slides were processed until the point that the autoradiographic film should be applied. Instead, the slides were submitted again to hybridization conditions in which each slide was incubated with 0.022 μg [^{3}H]cRNA (152,928 cpm/slide) in 50% formamide, 2 × SSC for 60 min at 20 ± 1°.

In all instances the RNA solution applied was recovered as described and the radioactivity determined. In order to establish the background binding of [^{3}H]cRNA, slides containing non-denatured chromosomes were submitted to the same hybridization procedure. The RNA solution was recovered and the reduction of the radioactivity measured. The loss of radioactivity varied from 1000 to 1,300 cpm per slide. The effect of the RNAase treatment following hybridization was investigated by counting the radioactivity in the washes following the treatment. The radioactivity released from the slides varied from 400 to 500 cpm per slide.

Since the total number of counts involved in background binding and "mismatching" is low as compared to the number involved in hybrid formation (table 1) and the variations in the numbers obtained from different slides are small, the data presented in table 1 show that during incubation at 65° without

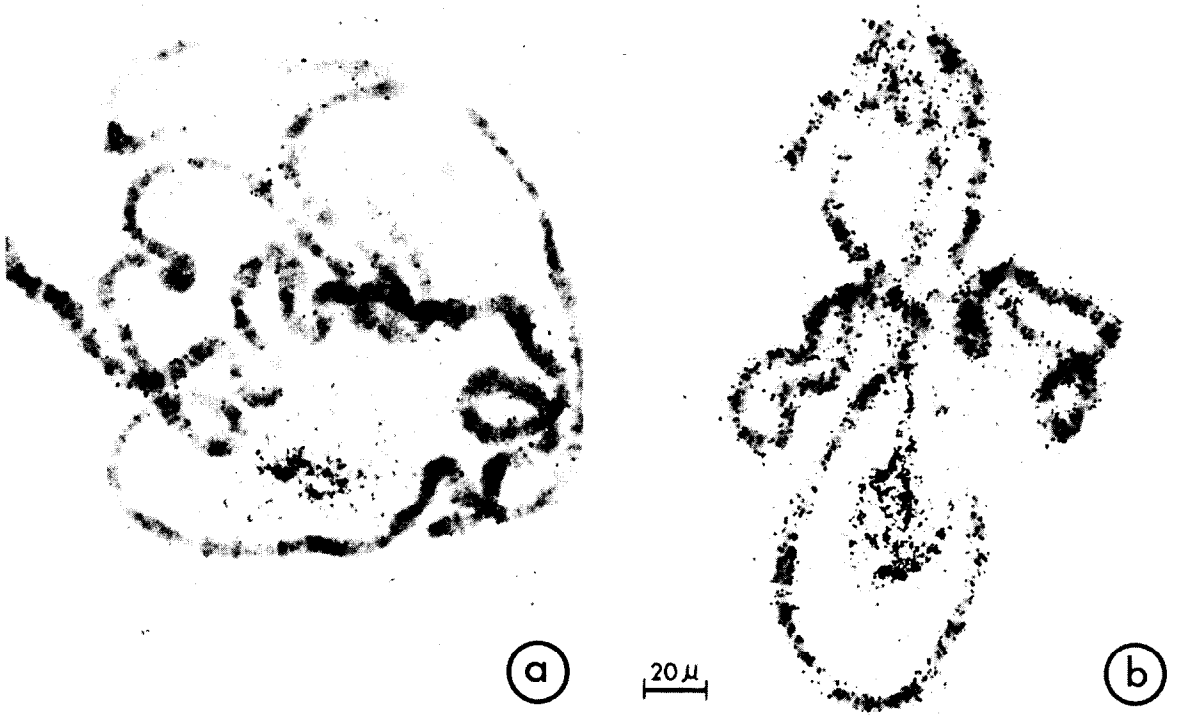

Fig. 1. Autoradiograph of *Drosophila hydei* salivary gland chromosomes after *in situ* hybridization of total RNA synthesized *in vivo* (3×10^6 dpm/slide). a) Hybridization at 65° in 6 × SSC for 12 hr. Exposure time 62 days. b) Hybridization at 20° in 50% formamide, 2 × SSC for 12 hr. Exposure time 62 days.

RNA there is a gradual reduction with time of the availability of DNA sequences which hybridize with cRNA. In slides submitted to sham hybridization conditions using formamide only a very small reduction was observed.

These results are compatible with the idea that during incubation at 65° the DNA gradually reassociates, whereas reassociation seems to be prevented to a large extent in the presence of formamide. The possibility that some DNA is lost during incubation at 65° cannot be excluded.

The higher relative effectiveness of the hybridization reaction carried out at 20° in the presence of formamide was also obvious from the autoradiographical analysis of the binding of [^{3}H]cRNA, total *in vivo* synthesized [^{3}H]RNA and pooled 18 + 28 S [^{3}H]RNA. In all instances, in which the quantity of [^{3}H]RNA applied, the hybridization time and the exposure time of the autoradiographs were identical, the hybridization reaction appeared to have occurred with much greater relative effectiveness in the presence of formamide at 20° than in its absence at 65° (fig. 1).

Hybridization of pooled 18 + 28 S RNA was restricted to the nucleolus organizer located within the nucleolus in the species used (fig. 2) [16].

It may be concluded that the hybridization procedure reported here eliminates a number of disadvantages inherent in the usual procedure.

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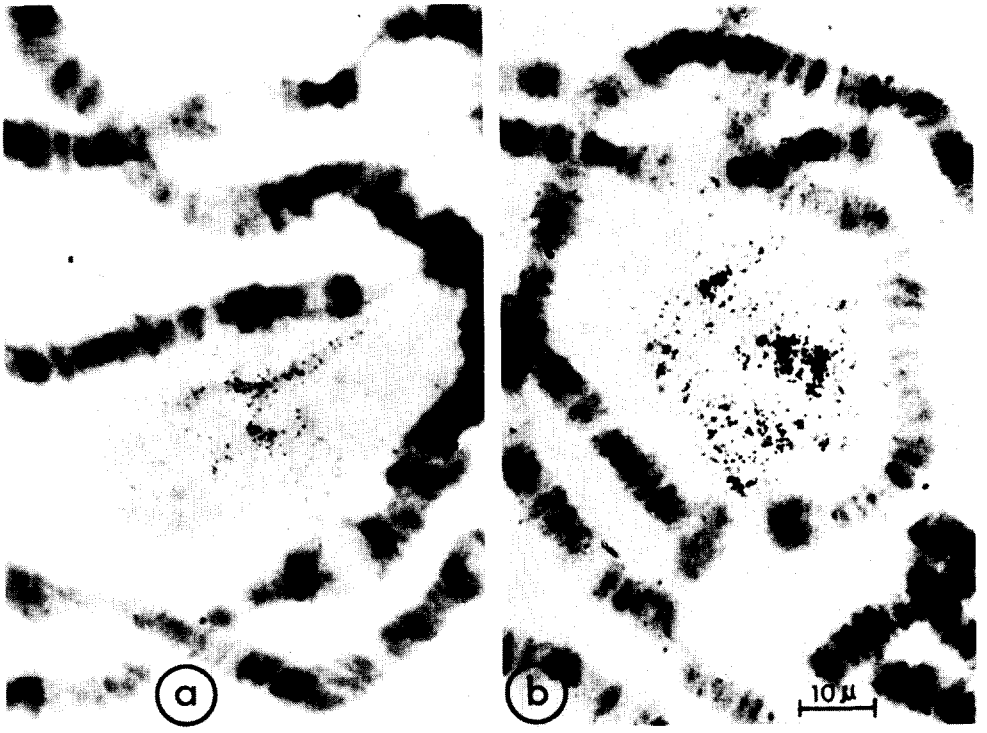

Fig. 2. Autoradiograph of *Drosophila hydei* salivary gland chromosomes after *in situ* hybridization of pooled 18 + 28 S RNA (3.6×10^6 dpm/slide). a) Hybridization at 65° in 6 × SSC for 12 hr. Exposure time 70 days. b) Hybridization at 20° in 50% formamide, 2 × SSC for 12 hr. Exposure time 70 days.

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