Influence of Temperature on the Detectibility and Chromosomal Distribution of Specific DNA Sequences by in situ Hybridisation

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Abstract. Hybridising certain AT-rich satellite complementary RNAs (cRNAs) to their homologous chromosomal DNA sequences at different temperatures of incubation results in a different dispersion of autoradiographic label throughout the karyotypes. The temperature at which most label, or cRNA-DNA hybrid formation, exists corresponds to the optimal rate temperature for the hybridisation of these same satellite cRNA-DNA hybrids as determined by RNA excess filter hybridisation. It is likely that the in situ hybridisation results can therefore be explained by the fact that there is a similar temperature-dependence on the rate of hybrid formation for both in situ and RNA excess hybridisation. This should have important implications for the designing of in situ hybridisation experiments in general.

Introduction

The in situ hybridisation technique, which combines the specificity of molecular nucleic acid hybridisation with cytological discrimination, possesses several features which compare with conventional hybridisation techniques, particularly RNA excess hybridisation (Jones, 1973; Hennig, 1973). RNA excess hybridisation reactions are known to have optimum rate temperatures (Birnstiel et al., 1972) which are influenced by a variety of factors including the GC content of the RNA, the RNA molecular length, and possibly the RNA conformation. At hybridisation temperatures above this optimum the amount of potential hybrid, or the reaction's saturation value, may be reduced. For the most part, in situ hybridisation reactions have been performed according to the procedures adopted for high GC ribosomal RNAs as reported by John et al. (1969) and Gall and Pardue (1969) where the temperatures of hybridisation were well below both the optimal rate temperature and the thermal dissociation range for the hybridisation and melting of the RNA–DNA hybrids (see Birnstiel et al., 1972 for example). At temperatures such as these, however, not all species of RNA–DNA hybrids may be fully formed. AT-rich DNA, for example, denatures at lower temperatures than GC-rich DNA and many AT-rich satellite DNAs have low thermal stability (Bond et al., 1967; Corneo et al., 1973). Moreover, RNA–DNA hybrids are frequently less stable than the corresponding DNA–DNA duplex with the same base-composition (Chamberlin, 1965; Walker, 1969; Bishop, 1972): for instance, U-containing polymers melt at very much lower temperatures than their T-containing analogues (Walker, 1969).

Because of this, many in situ hybridisation reactions, particularly those involving AT-rich DNAs, may have underestimated the amount of potential hybrid that can form at the cytological level. Thus it is important to determine
whether or not in situ hybridisation reactions have a temperature-dependent factor and whether such a temperature dependence might reflect a basic underlying similarity between this method and other conventional hybridisation methods.

In order to study this, we have investigated the hybridisation of certain AT rich satellite cRNAs to their homologous DNAs both by conventional RNA excess and in situ hybridisation. The results show that the in situ hybridisation reaction is dependent on temperature, and further, consideration of this temperature effect when designing in situ hybridisation reactions may well increase their efficiency.

Materials and Methods

1. Cells and Cell Culture Technique. Chromosome preparations from mouse embryo cells (J. B. T/Jd) and normal human lymphocytes were made as previously described (Jones, 1970; Jones and Corneo, 1972).

2. DNA Preparations. Mouse liver and human placental DNA was obtained by the method of Marmur (1961) with the inclusion of repeated phenol-chloroform, RNAse and Pronase treatments. Satellite and main band DNA were separated by preparative density gradient centrifugation in Ag⁺-Cs₂SO₄ (Jensen and Davidson, 1966). Mouse satellite DNA was purified by the method of Corneo et al. (1968); and human satellite DNAs I, II, and III prepared by the methods of Corneo et al. (1970, 1971). After exhaustive dialysis against high salt (5M NaCl) to remove the metal ions a sample of the satellite DNA was centrifuged in neutral CsCl, in the Beckman Model E analytical ultra-centrifuge for 24 hours at 44,000 rev./min at 25°C to ascertain satellite purity. Ultraviolet photographs were taken and traced on the Joyce-Loebel microdensitometer. The buoyant densities of the different satellite DNAs were determined from the position of a marker: Micrococcus luteus DNA, buoyant density 1.731 g/cm³ in neutral CsCl. Alkaline CsCl gradients were prepared according to Flamm et al. (1967) with the inclusion of Micrococcus luteus DNA, alkaline buoyant density 1.788 g/cm³ (Vinograd et al., 1963). A trace amount of highly purified mouse satellite DNA was added to 3.3 ml of a 0.01 M Tris-HCl (pH 8.5) solution containing 40 μg Xenopus DNA and 20 μg Micrococcus luteus DNA: 100 μl 1N NaOH were added, followed by 500 μg sodium lauryl sulphate (SLS). The solution was finally brought to an initial density of 1.760 g/cm³ with CsCl (BDH, analytically pure) and the DNA centrifuged in the MSE 50 rotor for 40 hours at 44,000 rev./min at 25°C. The separated strands of mouse satellite DNA banded in alkali at 1.762 g/cm³ (H) and 1.738 g/cm³ (L) respectively, Xenopus DNA banded in alkali at 1.754 g/cm³ (all determined in the analytical ultracentrifuge).

3. Preparation of Complementary Satellite RNA. High molecular weight, purified satellite DNA was exhaustively dialysed against 0.001 M NaCl before transcription. The cRNA was transcribed from 1μg native DNA template using E. coli DNA-dependent RNA polymerase, prepared as described before (Chamberlin and Berg, 1961). The incubation mixture containing 0.1 M Tris pH 7.5, 0.5 mM MnCl₂, 4 mM MgCl₂, 1.6 mM Spermidine, 70 mM KCl, 2 mM K₂HPO₄, 5 nmoles each of 3²HATP, ³²HUTP, ³²HCTP, ³²HGP all 15-29 CI/m mole, Amersham), and finally 2.5 units of enzyme. The final reaction volume was 0.1 ml. Incubation was carried out for 30 minutes at 37°C and the product cRNA was purified and isolated as described before (Jones, 1973). The RNA, made up to the appropriate salt concentration for hybridisation reactions, usually 3 × SSC or 6 × SSC 30% Formamide (FA), had a specific activity of 1.4-2.0 × 10⁷ cpm/μg.

4. Hybridisation Procedure. Denatured DNA was loaded onto filters according to the method of Gillespie and Spiegelman (1965). 0.002 μg total mouse DNA or 0.1 μg-0.02 μg total human DNA were usually bound to each filter along with 3.0 μg carrier bacterial DNA. Before hybridisation, the filters containing the bound DNA were soaked in the reaction medium minus RNA; generally 3 × SSC or 6 × SSC 30% FA (see below). Temperature optima (Tₒₜ) for individual hybridisation reactions were determined by the method of Birnstiel et al. (1972), the reaction not exceeding 30% of the saturation value. For kinetic studies, the hybridisation medium was brought to optimal temperature, filters introduced and individual ones