THE NATURE OF THE VARIABLE DNA ASSOCIATED WITH ENVIRONMENTAL INDUCTION IN FLAX

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SUMMARY

Large heritable differences in plant weight and in nuclear DNA amount can be induced in certain varieties of flax. The difference in DNA between the two stable types has been examined by analytical ultracentrifugation, thermal denaturation and renaturation kinetics. No differences in any localised nucleotide sequences are detectable by these methods. It is concluded, therefore, that the additional DNA found in L is representative of a wide spectrum of the flax genome in terms of composition, repetition and complexity of nucleotide sequences.

1. INTRODUCTION

HERITABLE changes in plant weight can be induced in the flax variety Stormont Cirrus by growing the plants from germination in specific fertiliser environments (Durrant, 1962, 1971). Two extreme forms, large (L) and small (S) stable genotrophs, result from the application of nitrogen and phosphorus respectively to plants grown in soil in a heated greenhouse. L is 3-6 times the size of S and the two behave as distinct genetic types. During the first 5 weeks of induction the specific treatments also induce a divergence of nuclear DNA content (Evans, Durrant and Rees, 1966; Evans, 1968) with L ultimately having 16 per cent more than S. The original parent (PI) is intermediate in both plant weight and DNA content. Other phenotypic effects also occur (Durrant and Nicholas, 1970).

The biochemical nature of the DNA difference produced during induction is of interest not only in understanding the mechanism of induction but also as a system for studying variation in possible flexible portions of the genome. We have already reported (Timmis and Ingle, 1973) that the rRNA genes are preferentially affected during induction, L having about 70 per cent more sequences coding for rRNA than S, and that this difference is correlated with nucleolar size in the two genotrophs. The difference in rRNA genes, however, only accounted for 0.23 per cent of the difference in DNA between the two genotrophs, so other sequences must also be involved.

In this paper we present the results of experiments designed to determine the molecular composition, complexity and degree of repetition of DNA sequences involved in the induced change.

2. MATERIALS AND METHODS

(i) *Preparation of DNA*

DNA was prepared from apical tissue of L and S plants (grown together in pots in a moderately heated greenhouse for 5-6 weeks) and purified by

preparative CsCl gradient centrifugation (Wells and Ingle, 1970). When low molecular weight DNA was required this DNA was hand sheared, using a syringe with a fine-bore needle. The size of the single stranded fragments was routinely estimated by sedimentation in 0.9 M NaCl and 0.1 M NaOH (Studier, 1965). Beckman Model E ultracentrifuge analysis of the DNA was performed using *Micrococcus lysodeikticus* DNA (1.731 g/ml) as a marker (Wells and Ingle, *loc. cit.*).

(ii) *Thermal denaturation*

Pure DNA samples (15-20 $\mu\text{g/ml}$) of L, S and *E. coli* DNA in $1 \times \text{SSC}$ (0.15 M NaCl + 0.015 M Na citrate, pH 7.2) were extensively dialysed against $1 \times \text{SSC}$ and heated in a Unicam SP 800 spectrophotometer fitted with a 4-cell temperature control block. The temperature was increased 1°C per 4 minutes and the OD_{260} of each cell measured every minute. The temperature was monitored by a thermistor in the fourth cuvette, containing $1 \times \text{SSC}$, at each reading. In some experiments the four cells were used to measure two samples of L and two of S DNAs enabling a statistical measure of the variation "within" and "between" samples to be made. A computer programme was used to make correction for the expansion of water and for differentiating the melting curve. A sum of the squares of the differences between the four cells was accumulated during the computation which enabled variance ratio tests for significance to be applied to the data.

(iii) *Renaturation kinetics*

Purified samples of DNA (20 or 400 $\mu\text{g/ml}$ in $1 \times \text{SSC}$) were denatured in SP800 cuvettes in a boiling-water bath for 10 minutes. The percentage hyperchromicity varied from 35 to 39 per cent with no consistent difference between L and S. The mean (37 per cent) hyperchromicity was therefore used in calculating the amount of renaturation which was followed optically, in the SP800 spectrophotometer at 60°C ($\text{TM} - 25^\circ \text{C}$) using either 1 cm or 1 mm light path cuvettes. The degree of renaturation was plotted against C_0t value (Britten and Kohne, 1968).

3. RESULTS

(i) *Analytical ultracentrifugation*

The nuclear DNA of flax is resolved by neutral CsCl equilibrium centrifugation into three fractions, the main band at 1.699 g/ml, a less dense satellite at 1.689 g/ml and a heavy shoulder at 1.710 g/ml (Ingle, Pearson and Sinclair, 1973). If all the additional DNA of L was localised in one of these three fractions it is possible that the percentage satellite and/or the amount of DNA in the heavy shoulder would vary between the two types. Fig. 1 shows microdensitometer scans of UV photographs of L and S DNA and there is an equal (15 per cent) amount of satellite in both. In 15 pairs of comparisons we have been unable to show any differences at this level, suggesting that the variable DNA is not limited to any particular fraction of G + C content.

(ii) *Thermal denaturation*

Model E analyses would only indicate any change in a gross fraction of DNA of particular C + G content. Sequences of a higher C + G content,

however, also require a higher temperature for denaturation than the lower C+G sequences and the detailed analysis of melting curves, which measure the melting temperature of particular sequences of DNA, allows more detailed comparison. The melting curve of *E. coli* DNA, a homogeneous DNA which melts over a small temperature rise of about 5° C. with a T_m

FIG. 1.—Microdensitometer scans of UV photographs of total DNA of L and S flax. Centrifugation at 44,000 r.p.m. at 25° C. for 20 hours in neutral CsCl in a Beckman Model E analytical ultracentrifuge. The buoyant densities were calculated relative to the internal marker DNA of *Micrococcus lysodeikticus* (1.731 g/ml) Main band: 1.699, Satellite: 1.689 g/ml.

of 93° C., is shown in Fig. 2. In contrast the DNAs of the L and S flax genotrophs are much more heterogeneous in C+G content, and melt over a wide range of about 15° C. There are several phases to the curve which is more easily analysed after differentiation of an average curve with the data meaned in pairs. This differential melting curve (Fig. 3) clearly resolves four main types of sequence varying in average base composition. There is a small fraction of low C+G content at 80° C. and a shoulder on the next mode at about 84° C. The first large mode itself, at 87° C., represents the first steep rise in Fig. 2. Melting analyses of purified flax satellite

DNA have confirmed that this represents the satellite component in the total preparation. The main band DNA shows a bimodal differential melt at 90° and 93° C., and several minor components melting at higher temperatures. The shape of the two curves is very similar in the two types. Small differences in the amount of DNA of particular compositions are present but are not statistically significant. It should be noted, however,

FIG. 2.—Melting curves of single samples of total DNA. DNA from *E. coli* (□) L flax (○) and S flax (●) in 1 × SSC. OD₂₆₀ readings were taken every 0.25° C. rise in temperature, which was increased 1° C. per 4 minutes.

that in three comparisons of melting profiles of L and S DNA there was always slightly more variation “between” than “within” the types, and small differences are possibly present.

(iii) Renaturation kinetics

The rate of renaturation of denatured DNA has been extensively used in studying animal genomes (Wetmur and Davidson, 1968; Britten and Kohne, *loc. cit.*) and has been particularly useful in studying the more simple homogeneous DNA sequences. Such analyses often indicate several classes of DNA sequences with different complexities and reiteration. For example, in *Drosophila hydei*, the highly reiterated satellite fraction (20 per cent of total with a $C_0t_{\frac{1}{2}}$ about 2×10^{-2}) is widely separated from the more complex unique sequences ($C_0t_{\frac{1}{2}}$ of about 10^2 ; Dickson, Boyd and Laird,

1971). With plant DNA the different regions of the genome, in terms of degree of repetition and complexity, are less well resolved. The genome appears to contain a heterogeneous mixture of sequences with a wide spread of repetition and complexity because the C_0t curves obtained are essentially linear (Cullis, 1973; Mitra and Bhatia, 1973).

FIG. 3.—Differential melting curves. The mean of two samples of L (○) and two of S (●) DNA in $1 \times \text{SSC}$. Data was meaned in pairs to give one reading every 0.5°C . rise in temperature.

Fig. 4 shows the rate of renaturation of L and S DNAs. The satellite sequences, which are highly repeated (Ingle, Pearson and Sinclair, *loc. cit.*), are not resolved from the rest of the genome, which must therefore be composed of sequences ranging from highly reiterated to unique. The low C_0t renaturations were replicated and the lines would therefore be expected to be coincident. This is the case in L (closed symbols) but not in S (open symbols) where there is a difference of about 8 per cent between the two replicates. This displacement is caused by variations in the hyperchromicity of different samples of DNA, a technical difficulty which was described in

"materials and methods". Variation in displacement of this order is caused by using the mean hyperchromicity of all DNA samples and is unavoidable in such experiments. Changes in sequences of particular multiplicity and complexity are indicated by differences in the shape of the curves. The shapes are very similar in the two types and it is not possible to attribute the DNA change to a class of sequences with a particular rate of renaturation.

FIG. 4.—Renaturation kinetics of L and S DNA. Denatured DNA, sheered to 0.7 and 0.5×10^6 Daltons single stranded molecular weight respectively, renatured in $1 \times \text{SSC}$ at 60°C . DNA at $20 \mu\text{g/ml}$: L ($\bullet$, $\blacksquare$) and S ($\circ$, $\square$); and $400 \mu\text{g/ml}$: L ($\blacktriangle$) and S ($\triangle$). The amount of renaturation was calculated using the mean percentage hyperchromicity (37 per cent) from these and other denaturations. C_0t value represents the product of the concentration of the DNA (Moles of nucleotides per litre) and the length of time in seconds during which the renaturation reaction has taken place.

4. DISCUSSION

Clearly the data obtained from analytical ultracentrifugation, thermal denaturation and renaturation suggest that the 16 per cent nuclear DNA difference during fertiliser treatments is not present in one particular fraction of the genome in terms of composition or repetition and complexity, but rather suggests that it is representative of a broad cross-section of the DNA sequences. This does not necessarily imply that all the sequences are increased or decreased equally otherwise "unique" sequences would have to be present in at least 5 or 6 copies. Rather the data suggest that some

sequences are increased preferentially but are not detected by the above procedures because their composition, repetition and complexity are wide ranging rather than restricted to any particular class. In addition, the sequences altered during induction from P1 to L may not be the same as those changed during P1 to S, which would reduce the level of change at the sequence level to about 8 per cent and further increase the difficulty in their characterisation.

The renaturation analyses contrast with those of Cullis (1973) who used hydroxyapatite chromatography to study renaturation of L and S DNA. His results suggested that changes in DNA sequences of intermediate degree of repetition may be responsible for the overall alteration in nuclear DNA. He further calculates that the extra sequences, derived by increasing some unique sequences, are represented in the genome at a multiplicity of about 70 times.

Hybridisation experiments (Timmis and Ingle, *loc. cit.*) have shown that L contains 3400 genes coding for rRNA compared with 2000 in S, which would only account for 0.23 per cent of the difference in DNA. Small changes in the buoyant density at which the genes banded in CsCl suggested that associated sequences also varied between L and S. Studies with a series of nucleolar mutants in *Xenopus laevis* (Amaldi, Lava-Sanchez and Buongiorno-Nardelli, 1973) showed that the mutants fell into three classes containing less nuclear DNA (88, 83 and 80 per cent) than the normal individuals. Hybridisation studies showed that the rRNA genes were differentially under-represented in the mutants in the same three classes, but again these genes accounted for only a small percentage of the total DNA change. However, they also demonstrated parallel changes in 4 S and 5 S RNA genes, from which they suggested that a portion of the genome was made up of families of repeated genes which were genetically regulated as a geometric series.

Certainly the number of rRNA genes present in particular species appears to be under genetic control. In hyacinth the number is affected by chromosomes, in addition to the nucleolus organising chromosomes of the complement (Timmis, Sinclair and Ingle, 1972) and a genetic control is also implicated by experiments using aneuploid lines of hexaploid wheat (Mohan and Flavell, 1974). In *Drosophila* the rRNA genes at the nucleolus organiser may be increased or decreased over very short periods depending on the chromosome constitution (Tartoff, 1971). In *Xenopus*, individuals with a reduced redundancy for rRNA genes are very frequent in natural populations and in the progeny of "normal" parents. The frequency of "mutation" in this latter cross is variable (Miller and Gurdon, 1970) with the variability ranging from 1 to 30 per cent (Amaldi *et al.*, *loc. cit.*) a level of change which is likely to be due to genetic or environmental factors other than mutation.

In flax it is known that not all varieties are able to show environmentally induced changes. Crosses between different varieties reveal that this ability is genetically controlled by interdependent nuclear and cytoplasmic factors (Durrant and Timmis, 1973). If genetic control mechanisms exist for the regulation of repeated families of genes it is not improbable that the system may be susceptible to the environment.

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