NUCLEAR PROTEINS OF DRY AND GERMINATING CONIFER SEEDS

J. A. Pitel and D. J. Durzan[1/]

ABSTRACT.--The proteins of the nuclear sap, the histones, and the nonhistone chromosomal proteins (NHCP) were extracted from a number of dry and germinating seeds and their composition was examined by polyacrylamide gel electrophoresis. The nuclear fraction was isolated and washed extensively with several buffer mixtures. The chromosomal material was then solubilized in a high salt-high urea buffer. After removal of the DNA by ultracentrifugation, the chromosomal proteins were passed through a QAE-Sephadex column to separate the histones from the NHCP. Gel patterns of the NHCP varied quantitatively during the early germination of jack pine and minor qualitative differences in protein complement were also detected. Differences in the profiles of the NHCP were found among species of the Pinaceae. Histones from coniferous seeds compared favorably with pea histones in classification and electrophoretic mobilities. The changes in histones in profiles from different species and with different stages of germination were due mainly to the heterogeneous FI fraction. The methods are suitable for studies of nuclear protein metabolism and genetic regulation and expression in tree improvement programs. Biochemical techniques for extracting and characterizing nuclear proteins are summarized.

The control of transcription (suppression or induction of selected genes) is now being pursued by many scientists. The structure and function of the nuclear proteins are important in this control, and polyacrylamide gel electrophoresis of proteins can become a useful diagnostic tool when dealing with quantitative and qualitative changes in genetic expression. For example, Bekhor et al. (1974a) suggested that the nonhistone chromosomal proteins (NHCP) could be fingerprinted to study chromosomal aberrations once functions are assigned to each of these proteins.

1/ Respectively, Department of the Environment, Canadian Forestry Service, Petawawa Forest Experiment Station, Chalk River, Ontario. KOJ 1JO, and Forest Ecology Research Institute, Canadian Forestry Service, 800 Montreal Road, Ottawa, Canada K1A OW5.

In this report we summarize methods to extract and characterize the nuclear proteins and present the known biological functions of proteins, especially those dealing with genetic expression. It is hoped that this study will eventually lead to methods to predict genetic expression (i.e., before it can be expressed visually) and to improve the genetic quality of conifers.

METHODS

Extracting and Characterizing Conifer Nuclear Proteins

To study the nuclear proteins in tissue, species specificity, and gene regulation, the extracted proteins should be pure, intact, and free from aggregation. We selected and modified methods to obtain high yields for extracting nuclear proteins. We avoided the use of denaturants, increased the rapidity of extraction, inhibited degradative enzymes, avoided contamination, and retained as much of the biological activity of these proteins as possible. Because nuclear proteins from storage tissues are especially difficult to purify (Bonner et al. 1968a, Grellet and Guitton 1973, Pitel and Durzan 1974), various combinations and extensions of the existing methodology were tried.

Numerous reports indicate the presence of protease in chromatin preparations and in the cytoplasm which can degrade histones (Paik and Lee 1970, Kincade and Cole 1966). Selective tissue-specific proteases confuse the estimates of qualitative and quantitative differences in composition (Hnilica 1972). Several protease inhibitors decrease the activity of these enzymes. These include 0.05M sodium bisulfite (Panyim et al. 1971), diisopropyl fluorophosphate (Hnilica 1972), $CdSO_4$ (Vaughan and Comings 1973), and phenylmethylsulfonyl fluoride (Wintersberger et al. 1973, Towill and Nooden 1973).

With the conifers we routinely used sodium bisulfite as a protease inhibitor. This was found to be especially needed for the germinating seeds. However, sodium bisulfite should be used with caution because base (pyrimidine) modification of chromatin RNA may occur. In some cells it may reduce the yield of nuclei and chromatin (Towill and Nooden 1973). To further decrease the activity of nucleases and proteases the buffers were at pH 8.0 (Jockusch and Walker 1974). The reagents we used were of the highest purity and were made fresh to prevent changes in the buffers that could affect the properties of the extracted proteins. For example, aged urea solutions form cyanate that could then react with amino acid sulfhydryl groups of proteins (Graziano and Huang 1971).

To prevent aggregation of the histone F3 molecules, 2-mercaptoethanol was included in the buffer during electrophoresis. Mercaptoethanol is also useful in reducing the binding of the histones to the NHCP (Levy et al. 1972).

EDTA, which can lead to losses of the arginine-rich histones (Stein and Borun 1972), was not needed nor used.

Chemicals that could irreversibly denature the proteins were avoided. Acid treatment to extract the histones from nuclei or chromatin before NHCP separation affects the structural properties of the NHCP (Graziano and Huang 1971). Phenol has been used to extract the nonhistone proteins (Teng et al. 1971); however, this reagent can irreversibly affect the native structure in some of the NHCP. Studies by Mischke and Ward (1975) showed that acid treatment or phenol changed the position of some of the NHCP during electrophoresis. Ionic detergents bind strongly to proteins and thus are not useful except for electrophoretic studies (Shirey and Huang 1969).

To help the dispersal of extra-nuclear material, without damaging the nuclei, Triton X-100 (nonionic detergent) was added at low concentrations (0.2 to 0.5 percent) (Sadowski and Steiner 1968, Panyim et al. 1971). Higher concentrations break nuclei and may cause partial solubilization and loss of nuclear proteins (D'Allesio and Trim 1968). Lower concentrations remove the outer nuclear membrane.

We used sodium deoxychloate (bile-salt anion) to remove cytoplasmic contaminants. It has detergentlike properties and solubilizes many sub-cellular components (Hadler et al. 1971) including the outer nuclear membrane (Monahan and Hall 1973). Low concentrations of about 0.001 M are needed because concentrations in the range 0.005 to 0.1 M can selectively extract the histones (Smart and Bonner 1971a, 1971b) and higher concentrations can cause partial lysis of nuclei.

The nuclear preparation should be washed with buffered media to remove contaminating proteins originating from the nuclear sap and cytoplasmic proteins that become adsorbed during isolation to the nuclei and chromatin. Generally, washing is done with buffered isotonic (0.14M) saline and with varying concentrations of Tris-HC1 buffers. Jockusch and Walker (1974) found that increasing the ionic strength of the buffer from 0.001M to 0.01M Tris increased the amount of NHCP extracted. Huang and Huang (1969) decreased the ionic strength at pH 8.0 during purification of their nuclear preparation. Stronger saline (0.3M) improved the removal of cytoplasmic contaminants but losses of NHCP can still occur (Hnilica 1972). At saline concentrations of 0.35M, some NHCP are extracted together with cytoplasmic proteins (Comings and Tack 1973). Kostraba et al. (1975) found that the 0.35M NaCl wash contained proteins similar to the cytoplasmic and nuclear sap proteins. This fraction included loosely bound NHCP that, after four washings, contained 43 percent of the total NHCP. Goodwin and Johns (1973) reported a possibility of some histone degradation products in the 0.35M NaCl extract. At higher salt concentrations more NHCP is removed; e.g., at 2.0M NaCl, 81 percent is extracted.

We also used, with varying degrees of success, extractions at low pH for the removal of cytoplasmic-contaminating proteins. Dick (1968) used pH 2.8 for purification of the histone fraction. We also included citric acid at 0.01M (pH 2.6) and aqueous ethanol.

The ratio of the nucleus to the cytoplasm is important. If this ratio is as low as 1:500 by volume there are greater chances of contamination (Evans and Ozaki 1973). Most methods use dense sucrose for purification of the chromatin preparations. The chromatin can be centrifuged through 1.7M homogeneous sucrose or through discontinuous sucrose (Bonner et al. 1968a, 1968b; Panyim et al. 1970). In our studies with conifer seed tissue, we repeated this process to ensure greater removal of the contaminants. In addition to sucrose purification, prolonged stirring of the crude chromatin in Tris-CH1 buffers solubilizes some contaminating material (Bonner et al. 1968a).

To avoid denaturing conditions for the extraction of the chromatin proteins, we separated them by dissociating the nuclear preparation in a high salt-high urea buffer. Essentially all chromatin proteins can be separated from DNA by dissociation in a 2.OM NaCl-5.0M urea buffer (Bekhor et al. 1974a, 1974b; Kleinman and Huang 1972). DNA can be removed by centrifugation for 18 hr at 60,000 RPM, by column chromatography (such as Bio-Gel A-50 as described by Graziano and Huang 1971), or if desired, by denaturing conditions such as selective hydrolysis, by partitioning with phenol (Shelton and Neelin 1971), or with DNase. In our studies we used ultracentrifugation to remove the DNA followed by separation of the histones from the NHCP with QAE-Sephadex. The use of ion-exchange to separate these two classes of proteins has resulted in preparations with less than 1 percent cross-contamination between the two (Levy et al. 1972). One can also use columns of Bio-Rex 70 (Levy et al. 1972), SP-Sephadex C-25 (Graziano and Huang 1971), hydroxylapatite in high salt and urea (MacGillivray and Rickwood 1974), and electrophoresis on SDS (sodium dodecyl sulfate). Our studies with SDS electrophoresis showed negligible contamination of the histones and NHCP with each other. If needed, 2 percent SDS can be used to solubilize the chromatin components.

Numerous other combinations can be used for solubilization such as 6M Urea-0.4M GuCl (Levy et al. 1972), urea alone (Mischke and Ward 1975, Pitel and Durzan 1974), 1MCaCl2 (Mohberg and Rusch 1969), and NaCl alone (Busch 1968, Wang 1967). However, several reports have shown that not all NHCP are removed with 2.OM NaCl (Levy et al. 1972, Kostraba et al. 1975). In the latter study, 19 percent of the NHCP could not be removed from DNA with 2.OM NaCl (buffered). Most of these proteins were released with 3M NaCl-7M urea. A method using cations to condense the chromatin was found better than the use of 2M NaCl (Flavell and Kemble 1974). The latter had more contaminating RNA. Methods using shearing of the chromatin help in further dissociation (Levy et al. 1972).

Numerous methods selectively extract the histones from nuclear preparations. Variations occur in pH (Hnilica 1972); sodium deoxycholate (Smart and Bonner 1971a); SDS; mixtures of urea, NaCl, and ethanol (Bolund and Johns 1973); various salt concentrations (Hnilica 1972, Bolund and Johns 1973); removal of Fl with 5 percent TCA or perchloric acid, ethanolic-HCl; tRNA and double-stranded DNA (Ilyin et al. 1972), and others. The use of urea in combination with NaCl appears to be a superior method for both dissolution of the chromosomal proteins and for retention of their biological functions.

Nuclear proteins from storage tissues are hard to purify. Fambrough et al. (1968) found significant amounts of contaminants in histone preparation from pea cotyledons. These had low electrophoretic mobility and were said to be acidic ribosomal proteins and NHCP. Histones can be contaminated by proteins from the mitochondria and nucleoli (Mohberg and Rusch 1970). Histones prepared by acid or salt dissociation may have small quantities of ribo- and deoxyribonucleotides (Greenaway and Murray 1973). Direct acid extraction of the nuclei or chromatin to obtain the histones may result in some contamination by the NHCP (Sadgopal and Bonner 1970a, 1970b; Wilhelm et al. 1971). High molecular weight NHCP have been reported to contaminate certain histone preparations (Levy et al. 1972, Graziano and Huang 1971).

The extracted NHCP fraction can be contaminated from several sources. For example, Goodwin and Johns (1972; 1973) and Johns and Forrester (1969) found contamination of the NHCP by the nonspecific adsorption of cytoplasmic and nuclear sap proteins to the chromatin during isolation. Hill et al. (1971) said that the NHCP may be in equilibrium with the soluble nuclear sap proteins under physiological conditions, e.g., NHCP turnover. It was difficult to determine precisely what belonged to the NHCP or to the nuclear sap. The NHCP were also found to be contaminated by the acidic nuclear membrane proteins (Harlow et al. 1972) to the extent of 11 percent (Suria and Liew 1974).

By considering these observations and introducing the modifications for coniferous tissues we have prepared the nuclear and cytoplasmic proteins in figures 1 to 4.

RESULTS AND DISCUSSION

Our results show that the nuclear proteins (histones, NHCP, and nuclear sap proteins) can be extracted from conifers and characterized by polyacrylamide gel electrophoresis. The purity and resolution of these proteins are comparable to those described for other plants and animals.

Problems of identification of proteins often arise by contamination, degradation, selective extraction, and aggregation. Some of these proteins may be in equilibrium with each other (e.g., NHCP and nuclear sap proteins) and thus may be hard to define precisely as belonging to one class or

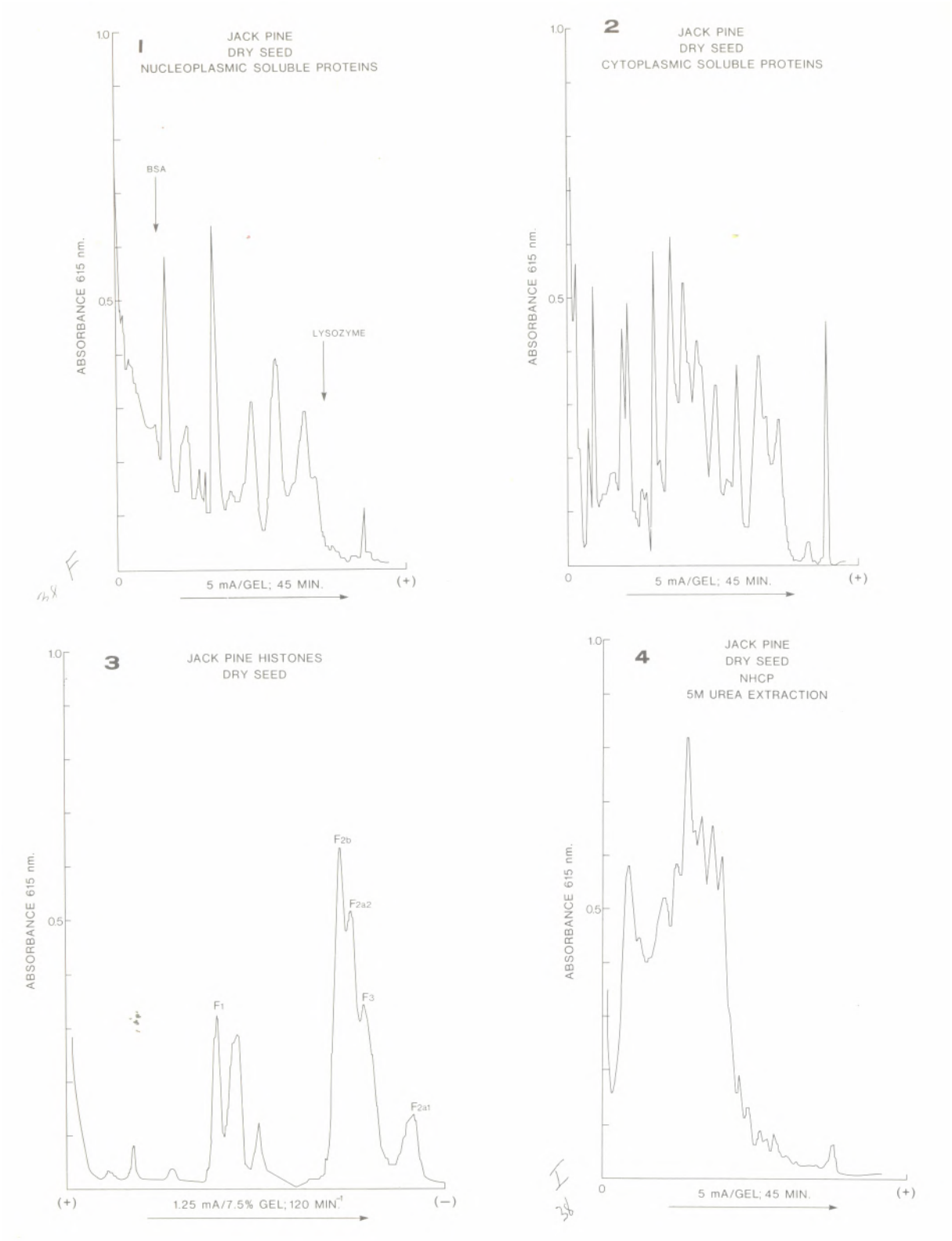

Fig. 1-4. Protein characterization of dry jack pine seed.

another. Also some can be transported between the cytoplasm and nucleus (Stein and Borun 1972). In our studies we examined the proteins of the cytoplasm, nucleoplasm, and the chromosomes and first compared them with each other to more precisely show that most belonged to separate classes and can be separated as such.

The cytoplasmic and nucleoplasmic soluble proteins had characteristic electrophoretic profiles that were significantly different from each other (figures 1 and 2). Differences in composition were found during germination (dry seed vs. 48 hr) for some of the bands for each of the classes. Studies with the cytoplasmic proteins, besides being useful to determine contamination with the different classes of proteins, are important because some of them may be gene regulators (Vaughan and Comings 1973), and may be able to bind to DNA (Choe and Rose 1974).

With jack pine we found that the soluble nuclear proteins were markedly different from those of the corresponding chromatin NHCP, although the two patterns showed some similar bands (figures. 1 and 4). Similar results were found by others (Comings and Tack 1973, MacGillivray and Rickwood 1974). Also, the nuclear sap proteins can bind to DNA (Vaughan and Comings 1973) and may have some gene regulatory function (Comings and Tack 1973).

Histones were extracted from various stages of germination of jack pine and found to vary quantitatively after 24 hours of germination (fig. 3). The profiles for the dry seed and for 12 hrs imbibed seed were identical. DNA synthesis is difficult to detect during early stages of germination (Pitel and Durzan 1975). Because histone synthesis is associated with DNA synthesis (Hnilica 1972), the lack of any major change in the histone profile is consistent with previous results. Changes in the histone fraction after 24 hrs imbibition were due mainly to the very lysine-rich (F1) fraction. The protease inhibitor, sodium bisulfite, was especially useful for the germinated seeds. Extraction of 4-day-old seeds in the absence of sodium bisulfite gave a 50 percent reduction in the content of the F1 histone and produced several additional bands in the F1 region and gave one band with a mobility between F3 and F2al .

Examination of the histones from several members of the Pinaceae revealed very little species specificity.

After passage of the conifer chromatin, soluble in 3M NaCl-5M urea, through a QAE-Sephadex column, the histone and NHCP fractions were analyzed by SDS electrophoresis and found to be essentially free of contamination from each other. By contrast, direct acid extraction of the nuclear preparation for histones gave numerous contaminating bands that could include proteins of the ribosomes (Mohberg and Rusch 1970, Fambrough et al . 1968) and NHCP (Graziano and Huang 1971, Grellet and Guitton 1973)

The NHCP were extracted using different concentrations of NaCl, various combinations of urea and NaCl, and urea alone. Profiles from all cases were largely similar on a qualitative basis. Most differences were found with the 0.14M NaCl. This extraction only removes 10 to 15 percent NHCP and is used mainly for removing nuclear sap proteins from the nuclei. Although this extraction contains the normal population of NHCP it also has proteins that resemble those from the nucleoplasm.

We compared the NHCP profile from dry jack pine seeds, where the chromatin is repressed, to that of the 48 hrs imbibed seed where gene activity was substantially greater. The major bands varied quantitatively. Unique bands were found with the minor proteins in germinated seeds. Although Coomassie Brilliant Blue was used for some studies it was not used to compare quantitative differences as the dye deviates from Beer's Law at higher protein concentrations (Chrambach et al. 1967). Caution must also be used with Amido Black, as it stains metachromatically (Johns 1967).

Limited species specificity was found when comparing the NHCP profiles of several Pinus spp. More qualitative and quantitative differences were found when comparing the Pinus spp. with those from Abies or Picea. The further interpretation of these results from a genetic and physiological viewpoint is in progress.

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