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Regulation of RNA Synthesis by DNA-Dependent RNA Polymerases and RNases during Cold Acclimation in Winter and Spring Wheat

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ABSTRACT

Chromatin DNA-dependent RNA polymerases and RNases activities were measured in winter and spring varieties to understand the overall regulation of RNA synthesis during cold acclimation. We found that total RNA polymerase activities were significantly higher in chromatin isolated from winter wheat compared to the spring wheat during the acclimation period. This increase was parallel to the increase in protein and RNA contents during hardening. The ratio of RNA polymerase I to RNA polymerase II activity was higher than 2 in winter wheat after 30 days of hardening compared, to a ratio of 0.90 under the nonhardening conditions. The increase in activity and the ratio of polymerase I to polymerase II was maintained after the separation of the enzymes from the template, suggesting that RNA synthesis is regulated in part at the enzyme level. On the other hand, the chromatin associated RNase activity decreased in both varieties during acclimation, indicating a nonspecific inhibition caused by low temperature rather than a selective genetic response associated with cold acclimation.

The enhancement of RNA and protein synthesis is one of the most important physiological changes in the hardy plant cell during the development of cold tolerance. It has been suggested that increased protein synthesis may result in the synthesis of cryoprotective substance(s) which increase membrane resistance to freezing and induce old hardiness (3, 4, 7, 10, 13, 14, 16, 27, 29). The mechanism by which cold hardening conditions regulate protein and RNA synthesis in hardy varieties is not fully known, but there is general agreement that frost hardening is associated with *de novo* protein synthesis. To understand the nature of this metabolic change, we studied the regulation of protein synthesis at the transcriptional level as a first step to elucidating the overall mechanism of regulation of cold hardiness. For this purpose, the functional activity of the chromatin was determined by measuring the change in DNA-dependent RNA polymerases and in RNase activities during hardening of two varieties of wheat differing in their degree of cold resistance.

MATERIALS AND METHODS

Plant Material and Cold Conditioning. Seeds of spring wheat (*Triticum aestivum* L. cv Glenlea) and winter wheat (*T. aestivum* L. cv Talbot) were placed in moist vermiculite and allowed to germinate for 5 d in the dark and 2 d under artificial light. Illumination of 450 μmol m^{-2} s^{-1} was provided by Sylvania F7212VHO/CW cool white fluorescent tubes plus 15% additional wattage as incandescent bulbs (Westinghouse). The temperature was maintained at 24 ± 1°C during the day and 20 ± 1°C during the night. The RH was 70 ± 5%. At the end of this period, control plants were maintained under the same conditions of light and temperature for 8 d. Hardening was performed for 42 d by subjecting the seedlings to a temperature of 6 ± 1°C during the day (10 h photoperiod) and 2 ± 1°C night. Evapotranspiration losses were replaced by daily addition of Arnon and Hoagland (2) nutritive solution.

Cold Hardiness Evaluation. Freezing resistance of seedlings from all treatments was determined as the temperature required to kill 50% of the plants (LD_{50}, °C) during a programmed 1°C h^{-1} decrease in temperature. Survival of plants was determined after a 2-week-regrowth period at 24°C day/20°C night (1, 20).

Extraction and Determination of Proteins and Nucleic Acids. Extraction and estimation of nucleic acids were carried out by the method of Howell (15). Commercial samples of yeast RNA and calf thymus DNA were used as standards.

Soluble proteins were extracted by homogenization of the tissues in 50 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 4 mM β-Mercaptoethanol, at 4°C with a Waring Blendor. After filtration and centrifugation at 105,000*g* for 3 h protein content was determined in the supernatant by the Lowry method (19) with BSA as the standard.

Measurement of *In Vivo* Nucleic Acids Synthesis. [^{3}H]Uridine (26.2 Ci/mol) and [^{3}H]thymidine (20 Ci/mol) from New England Nuclear were used to label the RNA and DNA, respectively. The seedling was carefully removed with the root system intact and washed thoroughly before incubation in the nutrient solution containing the radioactive uridine or thymidine at a concentration of 1 μCi/ml. Gramicidin D, 6 μg/ml (Calbiochem) was added to prevent bacterial growth during the experiment.

Extraction of nucleic acids was carried out as described above and aliquots of RNA and DNA fractions were counted in a Nuclear Chicago ISOCAP 300 liquid scintillation spectrometer to determine incorporation of the radioactive precursor.

Preparation of Chromatin. The tissues were homogenized in a Waring Blendor in 3 volumes per unit weight of: 0.5 M sucrose, 50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 4 mM β-mercaptoethanol, and 6 μg/ml gramicidin D. The homogenate was filtered through eight layers of cheese-cloth and two layers of Miracloth (Calbiochem), and the filtrate was centrifuged at 500*g* for 5 min. The pellet (crude chromatin) was suspended for 30 min in 1% Triton X-100 in 50 mM Tris-HCl (pH 8.0), 0.5 M sucrose, 25 mM KCl, 5 mM $MgCl_2$, 40% (v/v) glycerol, 1 mM DTT, and 6 μg/ml gramicidin D and the mixture was centrifuged at 15,000*g* for 15 min. The pellet was washed twice with 1% Triton X-100

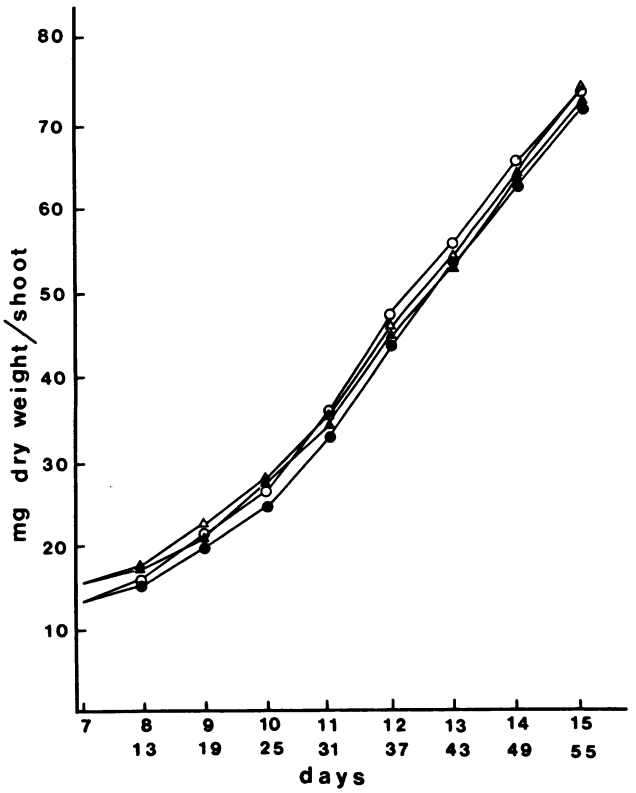

FIG. 1. Growth pattern of winter and spring wheat seedlings under hardening and nonhardening conditions. Values are expressed as the dry weight per shoot. ± SE for each value did not exceed 12%. (●), Winter wheat Talbot grown at 24/20°C (days 7–15); (○), winter wheat Talbot grown at 6/2°C (days 7–55); (▲), spring wheat Glenlea grown at 24/20°C (days 7–15); (△), spring wheat Glenlea grown at 6/2°C (days 7–55).

in the suspension buffer and once in the buffer alone. A purified chromatin pellet was collected by centrifugation and stored in the same buffer at −20°C.

DEAE Column Chromatography. The RNA polymerases from the chromatin fraction were solubilized and chromatographed on DEAE-Sephadex A-25 as described previously (22, 23).

Measurement of *In Vitro* RNA Synthesis. To estimate *in vitro* RNA synthesis, we determined the template activity of chromatin by measuring bound RNA polymerases under both growth conditions. Total RNA polymerase activity was measured in the standard RNA polymerase assay containing in 0.2 ml: 20 μg of chromatin, 0.05 M Tris (pH 8.0), 0.6 mM each of ATP, CTP, and GTP (Sigma), 0.06 mM of [^{3}H]UTP (2 μCi) (New England Nuclear), 1 mM DTT, and 10 mM $MgCl_2$. The reaction mixture was incubated for 20 min at 37°C, chilled, and precipitated with 10% TCA containing 20 mM sodium pryophosphate. The precipitate was collected on Whatman GF/A glass fiber discs, washed with 5% TCA-pyrophosphate, dried, and counted in Aquasol (New England Nuclear) in a Nuclear-Chicago liquid Scintillation spectrometer. RNA polymerase I activity was measured in the presence of 10 μg/ml α-amanitin and 20 mM $MgCl_2$, 50 mM KCl (optimal activity) and RNA polymerase II was calculated as the difference between the total polymerase activity and the activity of RNA polymerase I.

RNase Activity Measurement. Extraction and estimation of soluble RNases and chromatin-associated RNase were carried out as described previously (8, 9).

All experiments shown were carried out at least four times.

RESULTS

Growth and Cold Hardening. Winter wheat and spring wheat grown at 24/20°C for 15 d had growth patterns similar to the plants grown under the hardening conditions of 6/2°C for 55 d. Growth (as expressed per dry weight unit) per d at 24/20°C was comparable to growth per 6 d at 6/2°C (Fig. 1). On that basis, the cold hardened and unhardened seedlings were compared at similar growth stages. The winter wheat Talbot reached an LD_{50} of −20°C compared to −7°C for the spring variety Glenlea after 35 d of hardening.

Changes in Soluble Protein, RNA, and DNA Contents during Hardening. Total soluble protein content of shoots from cold-hardened plants increased for both cultivars as compared with that of shoots from unhardened seedlings. This increase was much more pronounced in winter wheat than in spring wheat (Fig. 2). At 55 d, the protein content of hardened winter wheat was 2.6-fold higher than that of unhardened plants. In comparison, spring wheat grown under hardening conditions showed a 30% increase compared to that grown under nonhardening conditions.

RNA and DNA contents of hardened winter wheat were found to be higher than the unhardened plants (Fig. 3). On the other hand, no change was found in RNA and DNA content in the spring variety grown under both conditions. Incorporation of [^{3}H]uridine and [^{3}H]thymidine in RNA and DNA, respectively,

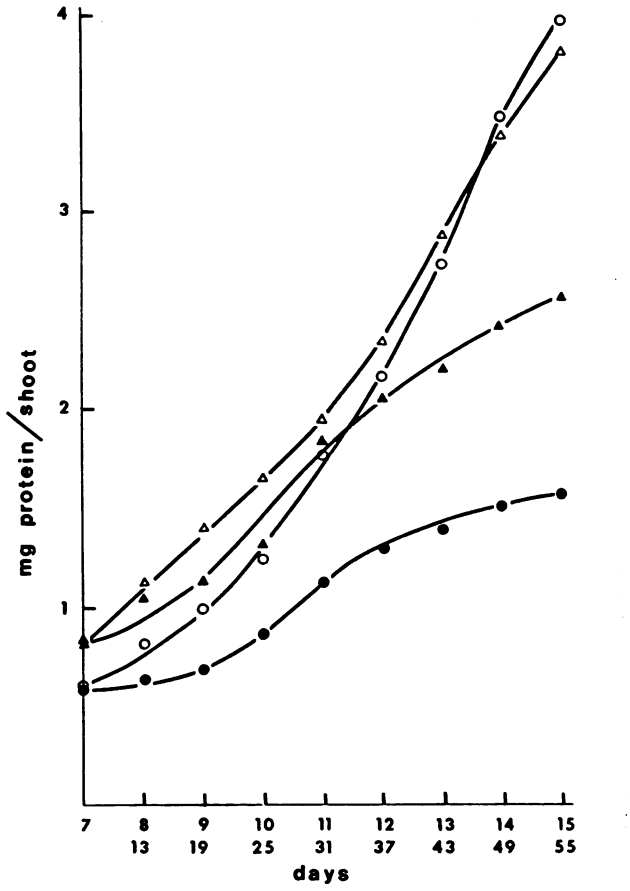

FIG. 2. Changes in total soluble protein contents in spring and winter wheat during hardening. ± SE for each value did not exceed 11%. (●), Winter wheat Talbot grown at 24/20°C (days 7–15); (●), winter wheat Talbot grown at 6/2°C (days 7–55); (▲), spring wheat Glenlea grown at 24/20°C (days 7–15); (△), spring wheat Glenlea grown at 6/2°C (days 7–55).

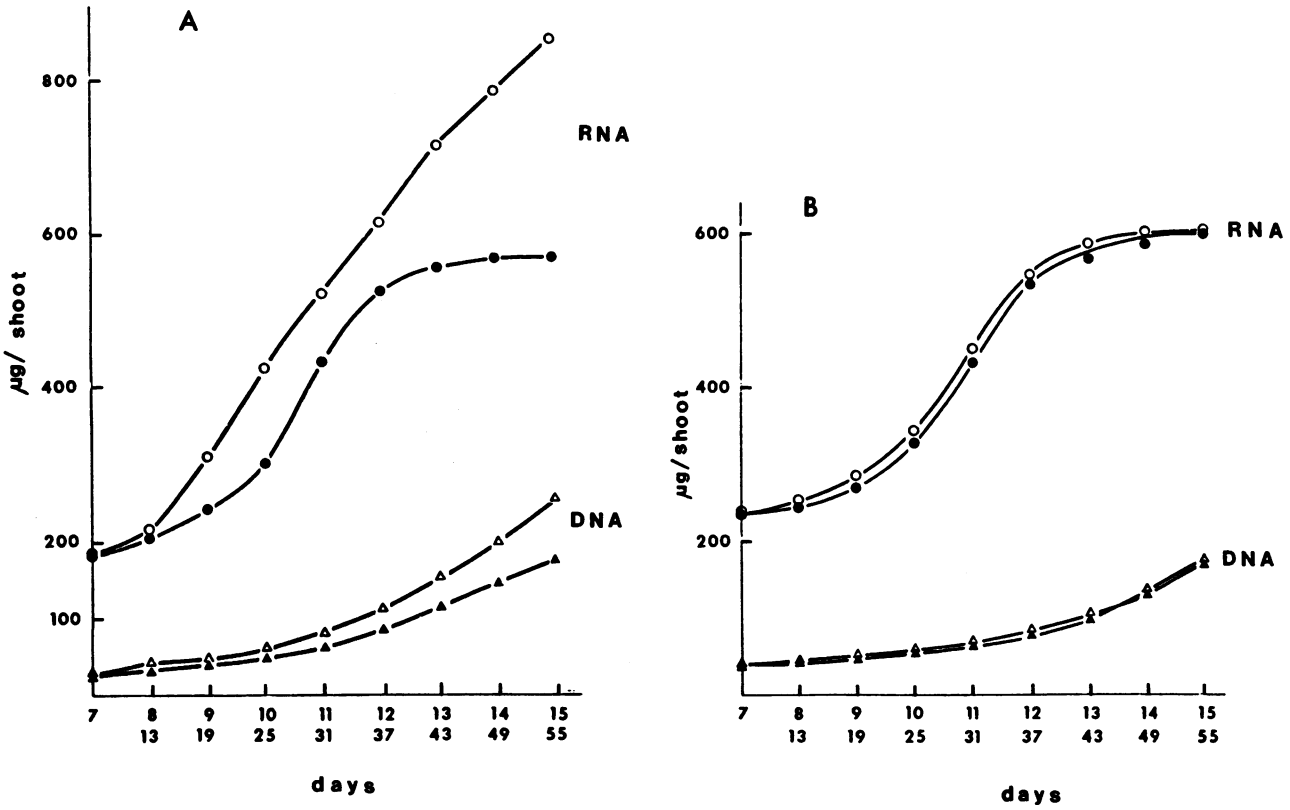

FIG. 3. Changes in total RNA and DNA contents in winter wheat (A) and spring wheat (B) during hardening. ± SE for each value did not exceed 14%. (●, ▲), Plants grown at 24/20°C; (○, △), plants grown at 6/2°C.

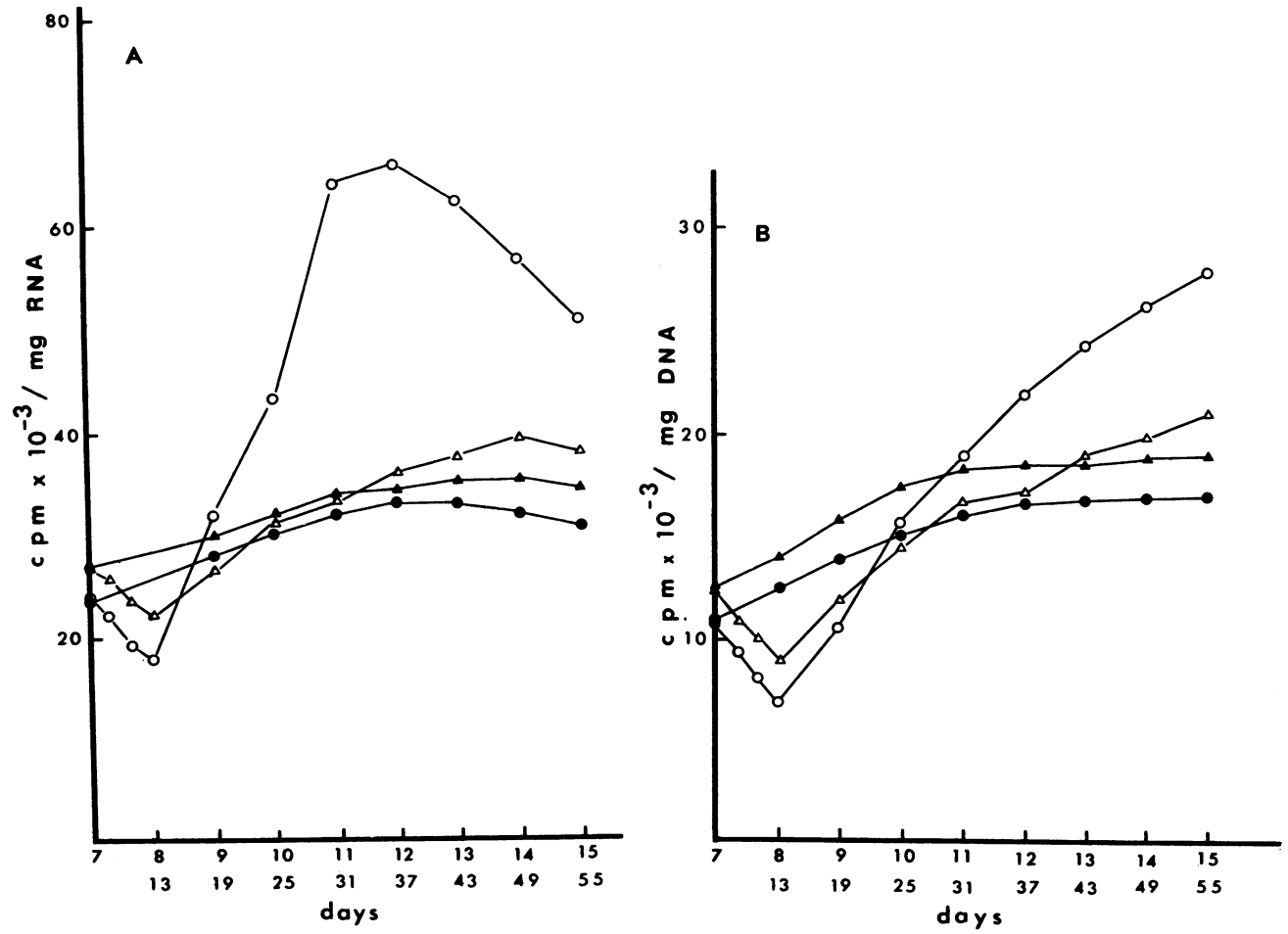

FIG. 4. Incorporation of [^{3}H]uridine into RNA (A) and [^{3}H]thymidine into DNA (B) in spring and winter wheat during hardening. ± SE for each value did not exceed 12%. (●), Winter wheat Talbot grown at 24/20°C (days 7–15); (○), winter wheat Talbot grown at 6/2°C (days 7–55); (▲), spring wheat Glenlea grown at 24/20°C (days 7–15); (△), spring wheat Glenlea grown at 6/2°C (days 7–55).

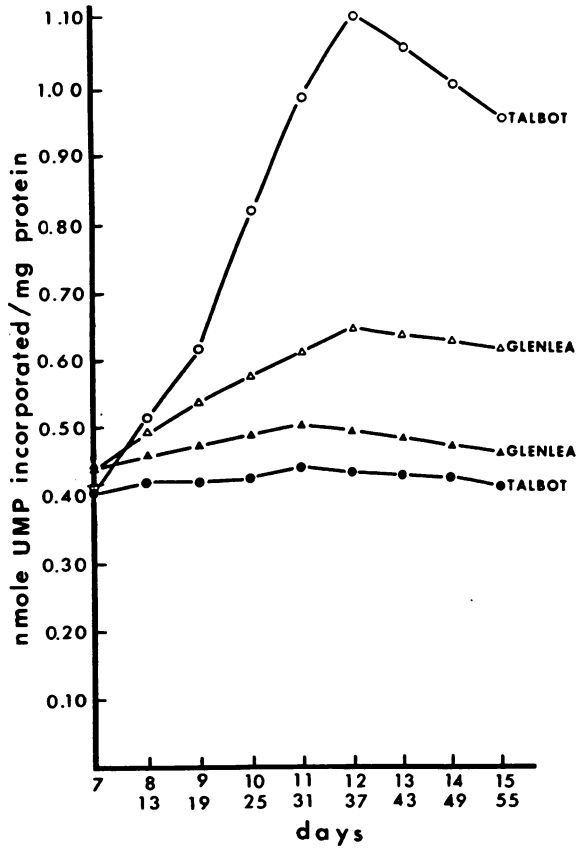

FIG. 5. Changes of chromatin-bound total RNA polymerase activity in spring and winter wheat during hardening. ± SE for each value did not exceed 14%. (●), Winter wheat Talbot grown at 24/20°C (days 7–15); (○), winter wheat Talbot grown at 6/2°C (days 7–55); (▲), spring wheat Glenlea grown at 24/20°C (days 7–15); (△), spring wheat Glenlea grown at 6/2°C (days 7–55).

was also found to be higher in winter wheat during hardening (Fig. 4). These results indicate that cold hardening of the winter variety is associated with *de novo* synthesis of RNA and DNA. It is interesting to note that we observed a decrease in uridine

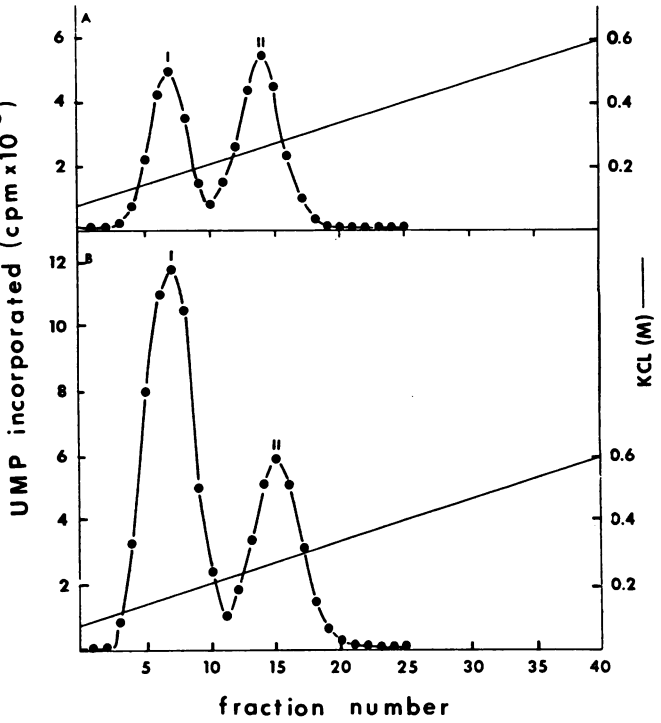

FIG. 7. DEAE-Sephadex A-25 column profile. The solubilized crude extract from winter wheat was chromatographed as described in "Materials and Methods." A, RNA polymerases I and II from plants grown at 24/20°C for 12 d; B, RNA polymerases I and II from plants grown at 6/2°C for 37 d.

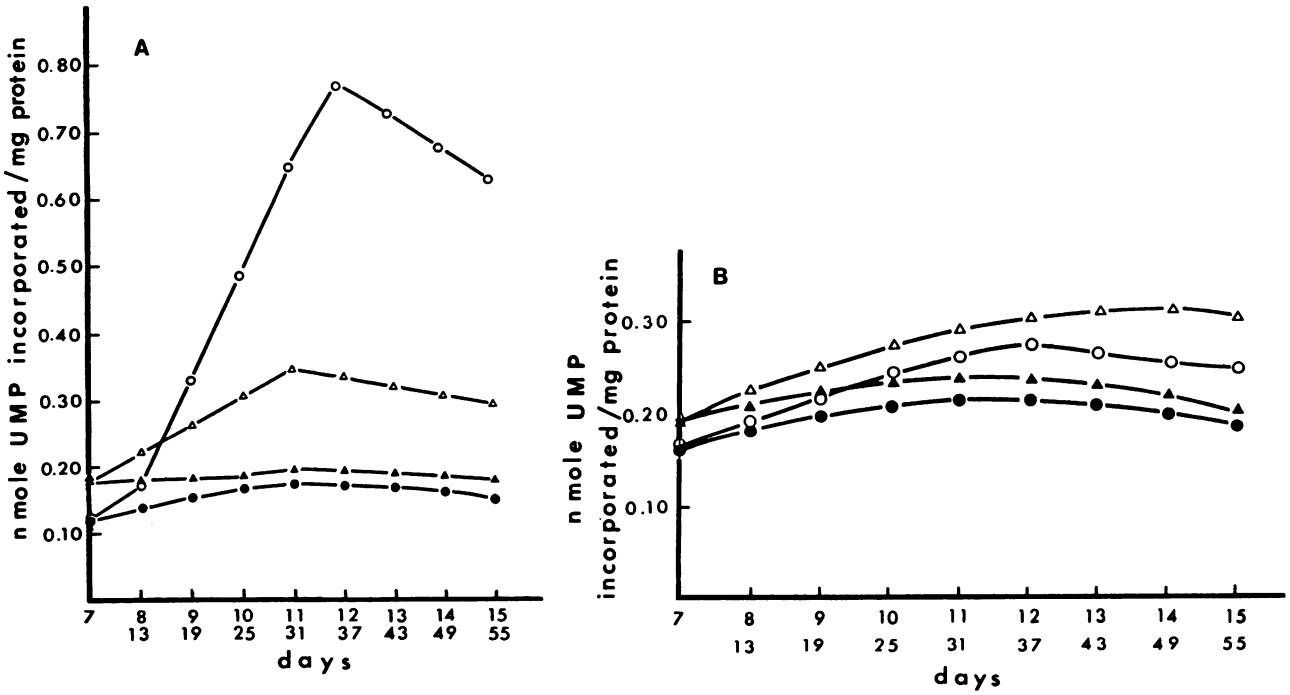

FIG. 6. Changes of chromatin-bound RNA polymerases I and II in the winter wheat Talbot (A) and spring wheat Glenlea (B) during hardening. ± SE for each value did not exceed 12%. (●), Polymerase I from plants grown at 24/20°C; (○), polymerase I from plants grown at 6/2°C; (▲), polymerase II from plants grown at 24/20°C; (△), polymerase II from plants grown at 6/2°C.

and thymidine incorporation during the 1st week of hardening in both varieties; this decrease could possibly result from the initial adjustment of plants to temperature stress.

Changes in RNA Polymerase and RNases Activities during Hardening. To determine which mechanism controls the enhancement of RNA synthesis during hardening, we examined the activities of chromatin-bound RNA polymerases and RNases in both cultivars under both growth conditions. The results (Fig. 5) indicate that the total chromatin-bound RNA polymerase activity was 3-fold higher in hardened winter wheat compared to the unhardened. The spring variety showed no significant changes under both growth conditions. This increase of the total RNA polymerases activities was mainly due to RNA polymerase I (α-amanitin insensitive) which increased 5-fold after 30 d of hardening compared to RNA polymerase II (α-amanitin sensitive) which increased 2-fold only (Fig. 6).

The ratio of RNA polymerase I to RNA polymerase II activity was higher than 2.2 in the winter variety after 30 d of hardening compared, to 0.90 in the nonhardening condition. These differences were still noticeable after chromatography of the solubilized enzymes on DEAE-sephadex (Fig. 7). These data suggest that increased RNA synthesis during cold hardening in winter variety is regulated in part at the RNA polymerases level. In contrast, the activity of chromatin-bound RNase was found to decrease during hardening in both spring and winter wheat (Fig. 8). This decrease in activity was independent of plant resistance and appears to be a response to low temperature.

Soluble RNase activities showed a 2-fold increase at the end of the hardening period in winter wheat compared to unhardened. In the spring variety, the difference between hardened and unhardened plants was less pronounced (Fig. 9).

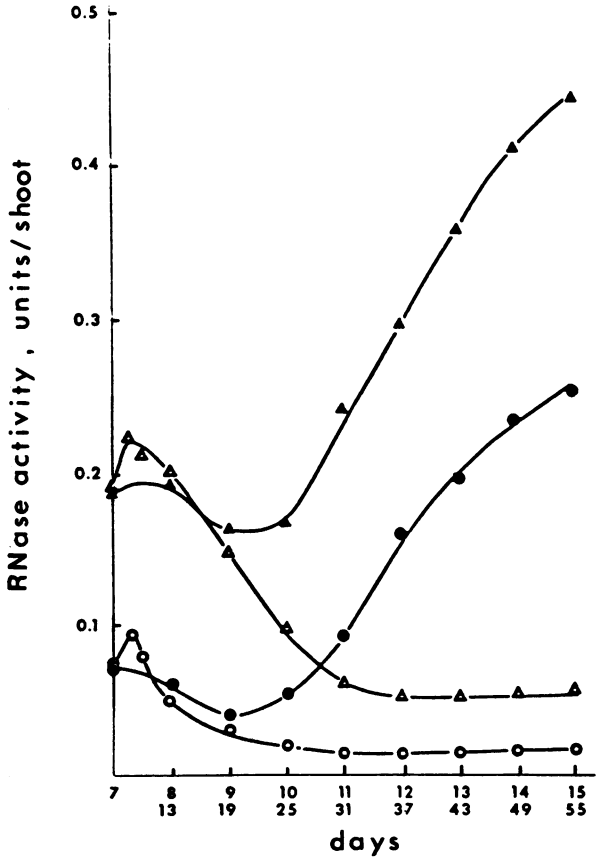

FIG. 8. Chromatin RNase activity in winter and spring wheat during cold hardening. ± SE for each value did not exceed 11%. (●), Winter wheat Talbot grown at 24/20°C (days 7–15); (○), winter wheat Talbot grown at 6/2°C (days 7–55); (▲), spring wheat Glenlea grown at 24/20°C (days 7–15); (△), spring wheat Glenlea grown at 6/2°C (days 7–55).

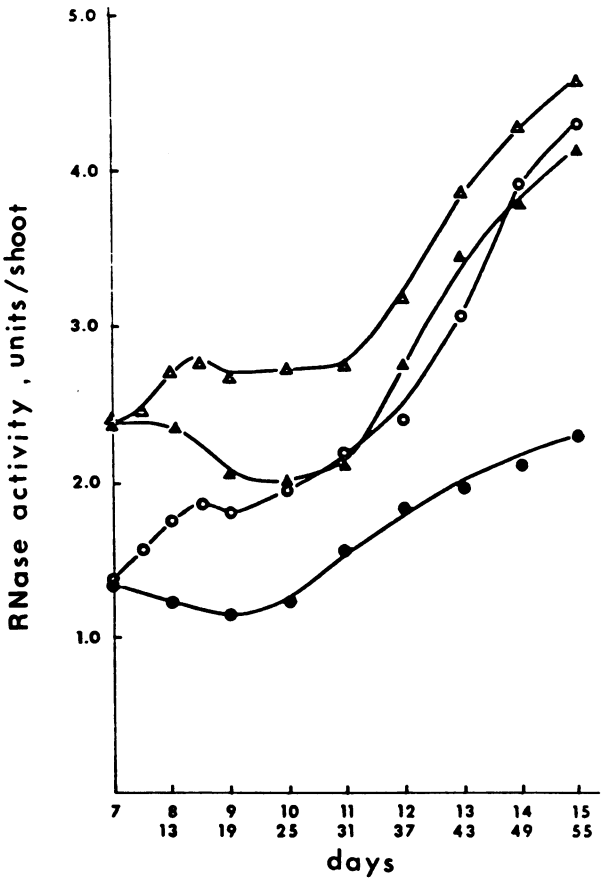

FIG. 9. Soluble RNases activity in winter and spring wheat during cold hardening. ± SE for each value did not exceed 13%. (●), Winter wheat Talbot grown at 24/20°C (days 7–15); (○), winter wheat Talbot grown at 6/2°C (days 7–55); (▲), spring wheat Glenlea grown at 24/20°C (days 7–15); (△), spring wheat Glenlea grown at 6/2°C (days 7–55).

DISCUSSION

Increase of protein synthesis activity, as judged by the changes observed in soluble protein, nucleic acids, and the functional activity of chromatin in hardened winter wheat, suggests that temperature during cold hardening induces a genetic response which enables these plants to develop a significant cold tolerance and withstand freezing stress during winter. The increase of uridine and thymidine incorporation indicates that RNA and DNA synthesis is associated with the increase in protein content and the development of cold hardiness. Although there is a general agreement concerning the increase of proteins and RNA during hardening (6, 7, 11, 17, 18, 24, 28), the increase of DNA is still to be clarified. Our results along with those of Shvedskaya, Teroak, and Kruzhilin (25, 26) showed an increase in DNA content, while other workers have found DNA content to be relatively stable during hardening (12, 18). This modest increase observed in DNA content could be due to increased copies of rDNA during cold hardening. However, an accurate measurement of rDNA copies should confirm this assumption.

The increase in RNA content and synthesis was found to be regulated, in part, at the transcriptional level. The enhancement of chromatin activity as measured by its capacity to synthesize RNA during hardening of winter wheat was regulated by DNA-dependent RNA polymerase I and II activities. RNA polymerase I was more active compared to RNA polymerase II suggesting that rRNA is synthesized at higher rate compared to mRNA.

These results may explain the increase in rRNA concentration observed during hardening in different plant systems (6, 12, 24). The increase in rRNA seems to be necessary for the plant to synthesize the proteins required for the cold acclimation process and it suggests that the *de novo* synthesis of rRNA is involved predominantly in the regulation of protein synthesis during this process. The stimulation of RNA polymerases activities seems to be regulated in part at the enzyme level since the solubilized enzymes showed an increased activity similar to that of the chromatin bound enzymes. The change in chromatin RNase activity did not seem related to the cold hardening process. The observed decrease in activity in both varieties is mainly due to the low temperature stress. The soluble RNases I and II increase during hardening were more pronounced in winter wheat as compared to spring wheat. These results are in disagreement with those of Gusta and Weiser (12) and Brown and Bixby (5) who found a decrease in soluble RNase activity during cold hardening. It is possible that the parallel increase RNases with that of RNA synthesis during hardening is directly related to RNA and protein turnover rates. It has been found that rapid RNA synthesis is associated with an increased in RNase activity during active growth. Phillips and Fletcher (21) concluded that the increase in RNase activity during RNA and protein synthesis is responsible for the rapid RNA turnover in *Phaseolus vulgaris*.

The accelerated synthesis of protein and RNA during hardening was associated with the marked increase in ATP level observed in our earlier study (20). This correlation indicates that the ATP level may be involved in the regulation of protein synthesis rate during hardening of the hardy variety. Our data suggest that the winter variety may have specific gene or set of genes which is expressed and regulated by the cold conditions of hardening. It is possible that the low temperature induces the synthesis of a hormone or of specific factors which regulate protein synthesis via the RNA polymerases and RNases and provide the energy requirement for the hardening processes. The products of these metabolic changes could be the factor(s) responsible for increasing membrane resistance to freezing and dehydration. The nature of these genetic responses and the specificity of the protein(s) synthesized during hardening will be clarified by studying the *in vitro* translation of mRNAs from cold-acclimated and nonacclimated winter wheat.

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