

Demonstration of transcriptional regulation of specific genes by phytochrome action

(photoinduction/isolated nuclei/transcriptional control/light-harvesting chlorophyll *a/b*-protein/ribulose-1,5-bisphosphate carboxylase)

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ABSTRACT We have developed an *in vitro* transcription system that uses nuclei isolated from *Lemna gibba* G-3. The *in vitro* transcripts include sequences homologous to hybridization probes for the small subunit of ribulose-1,5-bisphosphate carboxylase [3-phospho-D-glycerate carboxy-lyase (dimerizing), EC 4.1.1.39], the light-harvesting chlorophyll *a/b*-protein, and rRNA. Light-harvesting chlorophyll *a/b*-protein sequences are transcribed to a greater extent in nuclei isolated from plants grown in darkness with 2 min of red light every 8 hr than in nuclei isolated from dark-treated plants. Furthermore, the amount of these transcripts measured in plants given a single minute of red light after dark treatment is increased over the amount measured in dark-treated plants. The effect of red light is at least partially reversible by 10 min of far-red light given immediately after the red light pulse. Transcription of both rRNA and small subunit sequences is also stimulated by a single minute of red light as compared to dark-treated tissue. However, the relative magnitudes of the increases compared to the dark levels are smaller than the increase seen for the chlorophyll *a/b*-protein, possibly because of the higher level of transcription of these sequences in the dark. The effect of red light on the transcription of small subunit and rRNA sequences is also reversible by immediate treatment with 10 min of far-red light. Pulse chase studies of dark-treated nuclei for up to 110 min do not show substantial turnover of *in vitro* labeled small subunit and chlorophyll *a/b*-protein transcripts. We therefore conclude that phytochrome action has induced specific changes in transcription of these genes.

Phytochrome is involved in the light regulation of a number of plant responses. Recent interest has centered on the role of phytochrome in the light induction of two chloroplast proteins, the light-harvesting chlorophyll *a/b*-protein (LHCP) and the small subunit (SSU) of ribulose-1,5-bisphosphate carboxylase [3-phospho-D-glycerate carboxy-lyase (dimerizing), EC 4.1.1.39]. The genes for these two chloroplast polypeptides are encoded in nuclear multigene families (1–8). The SSU and LHCP precursor polypeptides are first synthesized on soluble, cytoplasmic polysomes, and they are taken up into the chloroplast posttranslationally and then cleaved to yield the mature forms (9–14).

Early studies on the light induction of ribulose-1,5-bisphosphate carboxylase have shown that the increase in activity of this enzyme is due to increased amount of the protein (15–17). More recently, *in vitro* translation of isolated mRNAs was used to show that light stimulates an increase in SSU and LHCP mRNAs (12, 16, 18–22). In both *Lemna* (duckweed) and barley the rapid light-stimulated increase in SSU and LHCP mRNAs is under phytochrome control (22–25). Phytochrome is also involved in the regulation of the expression over a longer term of a number of nucleus-encoded RNAs, including those encoding SSU and LHCP, in

pea and mung bean leaves (26–28). There have also been reports that phytochrome action can induce the synthesis (29, 30) and accumulation (31) of ribosomal RNAs and ribosomes in mustard seedlings.

The establishment of a phytochrome-mediated effect of light on the amount of mRNA for the SSU and LHCP polypeptides raises the question as to whether phytochrome acts to affect primarily transcription or messenger stability. In the absence of a simple *in vivo* labeling system, isolated nuclei have become the method of choice for such studies (32). In this study, we have set up and characterized an isolated nuclei system from *Lemna gibba* to address the question of whether phytochrome influences the transcription of SSU and LHCP. Preliminary accounts of this work have been presented elsewhere (33, 34).

MATERIALS AND METHODS

Growth of Plants. *Lemna gibba* Linnaeus G-3 was cultured aseptically in E medium (23) supplemented with 1% sucrose and 3 μ M kinetin. Plants were grown either in constant white light or in a dark growth chamber with 2 min of red light per 8 hr at 27°C \pm 2°C as described (23). The red and far-red light treatments of dark-incubated plants were performed as described in (23).

Isolation of Nuclei. Nuclei were isolated by a modification of the method of Luthe and Quatrano (35). Plants were harvested 2 hr after appropriate light treatments, rinsed in distilled water, and patted dry on paper towels. Ten-gram samples were ground in a chilled mortar in 20 ml of ice-cold Honda buffer (35) for about 30 s and then diluted with an additional 50 ml of the same buffer. The homogenate was sequentially filtered through four layers of cheesecloth, one layer of Miracloth (Calbiochem), and an 80- μ m mesh (Nitex; Tetko, Monterey Park, CA). The filtrate was centrifuged in a Sorvall HB4 rotor at 5,850 \times g_{max} for 5 min and the supernatant liquid was discarded. The pellet was gently resuspended in Honda buffer and filtered through a single layer of Miracloth. The nuclei were finally purified on a discontinuous gradient of Percoll as described in ref. 35 and stored in liquid N₂. All dark-treated plants were harvested and nuclei were prepared under a dim green safelight.

RNA Synthesis in Isolated Nuclei. Transcriptional activity of isolated nuclei was assayed in 60- μ l reaction mixtures. Each assay contained 1–6 \times 10⁶ nuclei incubated with 0.5 mM each of ATP, GTP, and CTP, 2.4 μ Ci (1 Ci = 3.7 \times 10¹⁰ becquerels) of [³H]UTP (40 Ci/mmol, Amersham), 75 mM (NH₄)₂SO₄, and 2 mM MnCl₂. Incubations were at 30°C for 30 min. Aliquots (15 μ l) of the incubation mixture were spotted onto Whatman 3 MM discs and precipitated with trichloroacetic acid as described (35).

Preparative RNA synthesis was carried out in 1 ml. Incu-

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Abbreviations: SSU, small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase; LHCP, light-harvesting chlorophyll *a/b*-protein.

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bation mixtures contained $1-5 \times 10^8$ nuclei, 75 mM $(\text{NH}_4)_2\text{SO}_4$, 2 mM MnCl_2 , 0.5 mM each of ATP, CTP, and GTP, and 250–400 μCi of $[\alpha\text{-}^{32}\text{P}]\text{UTP}$ (3000 Ci/mmol, New England Nuclear). The assay mixtures were prepared under a dim green safelight and incubated in darkness for 30 min at 30°C . Ribonucleic acids were purified from incubation mixtures by the method of Groudine *et al.* (36).

Agarose Gel Analysis of *in Vitro* Labeled Transcripts. RNA was synthesized in a standard transcription mixture (30 μl) containing 1×10^6 nuclei and 40 μCi of $[\text{P}^{32}]\text{UTP}$. Transcripts were isolated by addition of 1% NaDodSO₄/10 mM Tris-HCl/5 mM EDTA, pH 7.4, followed by extraction with phenol. Nucleic acids were precipitated by addition of 2.5 vol of ethanol at -20°C and then collected by centrifugation in a Sorvall SS34 rotor at $30,000 \times g_{\text{max}}$ for 30 min. Samples were fractionated in a 1% agarose gel in 20 mM sodium 4-morpholinepropanesulfonate/1 mM EDTA/5 mM sodium acetate/2.2 M formaldehyde, pH 7.0, and the gel was dried and exposed to preflashed x-ray film (Kodak XAR).

Hybridization of Cloned Probes to Nuclear RNA Labeled *in Vitro*. Three cloned probes were used to analyze *in vitro* labeled transcripts. The first, pLgSSU1, is a *Lemna* SSU cDNA inserted into the *Pst* I site of pBR322 (24). The second clone, pLgAB19/H5c, a subclone of a genomic clone, λAB19 , contains a sequence complementary to mRNA for LHCP inserted into the *Hind*III site of pBR322 (24, 34). *Lemna* rRNA (25S) sequences were analyzed by using pLg52, a cDNA clone constructed by W. J. Stiekema as described (24) and containing a 500-base-pair insert at the *Pst* I site of pBR322 (unpublished data). Restriction digests of pLgSSU1 with *Pst* I and of pLgAB19/H5c and pLg52 with *Hind*III were performed according to the supplier's recommendations (Bethesda Research Laboratories) to release the cloned, inserted sequences or to linearize the plasmid (pLg52). Fragments from 1 μg of *Pst* I-digested pLgSSU1, *Hind*III-digested pLgAB19/H5c, and *Hind*III-digested pLg52 were separated on 1% agarose gels in 90 mM Tris/90 mM boric acid/0.1 mM EDTA, pH 7.0, and blotted onto nitrocellulose by using the method of Southern (37). The filters were then challenged with RNA labeled in the *in vitro* transcription system. RNA-DNA hybridization was performed in 50% (vol/vol) deionized formamide/0.75 M NaCl/0.075 M trisodium citrate/10 \times Denhardt's solution (38)/100 μg of yeast tRNA per ml/10 μg of poly(U) per ml/10% (wt/vol) dextran sulfate as described by Thomas (39) for 3 days. Filters were washed 5 times, 15 min each, in 0.3 M NaCl/0.03 M trisodium citrate/0.1% NaDodSO₄ at room temperature, then 30 min at 60°C in 0.015 M NaCl/0.0015 M trisodium citrate/0.1% NaDodSO₄. After treatment with RNase A at 5 $\mu\text{g}/\text{ml}$ in 0.3 M NaCl/0.03 M trisodium citrate for 30 min at room temperature, filters were air dried and exposed to preflashed x-ray film (Kodak XAR) with a screen (DuPont Cronex Quanta IIFAH).

RESULTS

Characterization of Isolated *Lemna* Nuclei. With the method of Luthe and Quatrano (35), 10 g of constant white light-grown *Lemna* fronds yields about 10^{10} nuclei. In these isolated nuclei, $[\text{P}^{32}]\text{UTP}$ is incorporated into trichloroacetic acid-insoluble material in a linear fashion for at least 30 min (Fig. 1). The ammonium sulfate and manganese chloride optima were found to be broadly similar to those reported for wheat embryo nuclei (40) and isolated RNA polymerases I and II (41, 42). It is clear that the incorporation of $[\text{P}^{32}]\text{UTP}$ in this system is due to RNA synthesis rather than end-labelling of preexisting RNA because omission of ATP, GTP, and CTP or addition of actinomycin D abolishes incorporation (Table 1). Inclusion of the transcription inhibitor α -amanitin at 100 $\mu\text{g}/\text{ml}$ results in about 60% reduction in incorporation, indicating that about 40% of the incorporation in nuclei from light-grown plants is due to RNA polymerase I activity. The

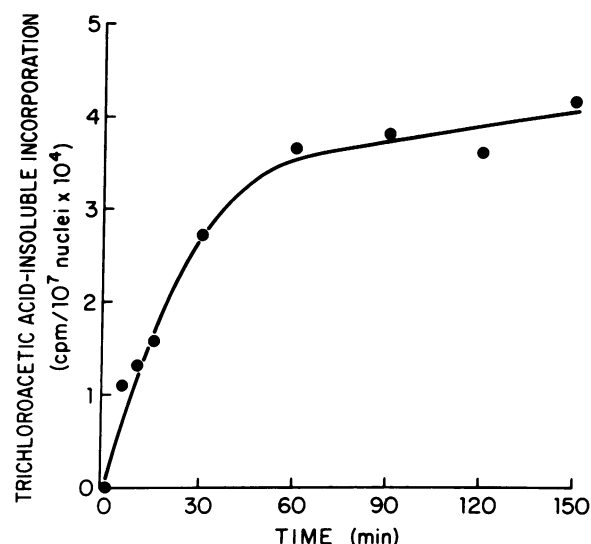


FIG. 1. Time course of incorporation of $[\text{P}^{32}]\text{UTP}$ by isolated *Lemna* nuclei. Duplicate aliquots (15 μl) were removed from an incubation mixture (300 μl) containing 2.3×10^7 nuclei, 25 μCi of $[\text{P}^{32}]\text{UTP}$, and other components as specified in the text, at the times shown.

pattern of inhibition by α -amanitin at the various concentrations indicates that all three RNA polymerases are active in this system (42). It is not known whether plant nuclei are capable of initiating RNA synthesis *in vitro*, and it is generally assumed that *in vitro* nuclear systems elongate transcripts initiated prior to nuclear isolation (32).

Analysis of *in vitro* transcripts by formaldehyde agarose gel electrophoresis shows that the RNA represents a continuous spectrum of sizes to greater than 25S (Fig. 2).

pLg52 Sequences Are Transcribed by RNA polymerase I. The effect of α -amanitin on the transcription of the cloned pLg52 sequences is shown in Fig. 3. RNA was synthesized in nuclei isolated from light-grown plants in the presence (section 3, lanes a–d) or absence (section 2, lanes a–d) of α -amanitin at a concentration (300 $\mu\text{g}/\text{ml}$) inhibitory to both RNA polymerases II and III and was hybridized to blots of the three clones or pBR322 alone. pLg52 sequences are transcribed in both the presence and absence of α -amanitin, whereas SSU and LHCP sequences are transcribed only in the absence of the inhibitor. This experiment demonstrates that pLg52 is transcribed by RNA polymerase I and gives an indication of the relative abundance of the rRNA and the SSU and LHCP gene transcripts in this system.

Role of Phytochrome in Control of Transcription of SSU and LHCP Genes. To investigate the role of phytochrome in the control of expression of SSU and LHCP genes, *in vitro*

Table 1. Characteristics of incorporation of $[\text{P}^{32}]\text{UTP}$ by *Lemna* nuclei isolated from white light-grown plants

Conditions	RNA synthesis	
	cpm	% of control
Control	36,444	100.0
– ATP, – GTP, – CTP	6,824	18.7
+ Actinomycin D (5 $\mu\text{g}/\text{ml}$)	4,516	12.4
+ α -Amanitin (0.1 $\mu\text{g}/\text{ml}$)	22,688	62.3
(5.0 $\mu\text{g}/\text{ml}$)	16,128	44.3
(100 $\mu\text{g}/\text{ml}$)	13,828	38.0

Reaction mixtures (60 μl) containing 1.15×10^6 nuclei, 75 mM $(\text{NH}_4)_2\text{SO}_4$, 2 mM MnCl_2 , 5 mM GTP, CTP, and ATP, and 2.5 μCi of $[\text{P}^{32}]\text{UTP}$ were incubated for 30 min at 30°C . Zero time incorporation of 778 cpm of trichloroacetic acid-insoluble material was subtracted from each value.

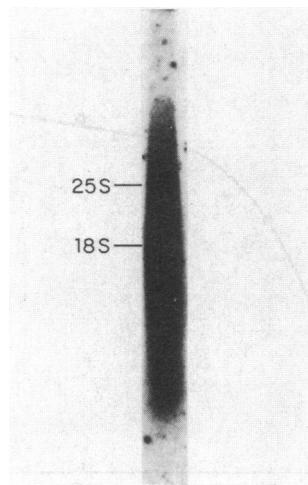


FIG. 2. Sizes of nuclear transcripts labeled *in vitro*. RNA transcripts labeled with [32 P]UTP were purified and fractionated on a formaldehyde agarose gel.

transcripts were prepared from nuclei isolated from plants grown under different light regimes. Nuclei were isolated from plants grown in darkness with intermittent red light (2 min of red light per 8 hr) for 7 weeks (Rc plants). Plants were also grown under this regime but placed in total darkness 7 days before harvesting (D plants). In addition, dark-treated plants were illuminated with either 1 min of red light (R plants) or 1 min of red light immediately followed by 10 min of far-red light (RFR plants) 120 min before harvesting. Southern blots of digested pLgSSU1 and pLgAB19/H5c were challenged with equal amounts of radioactive RNA from each preparation (Fig. 4).

Plants grown with intermittent red light transcribe substantial amounts of both SSU and LHCP sequences. After 7 days of darkness, the amount of SSU sequence transcribed decreases considerably and transcripts of LHCP sequences are barely detectable. However, 1 min of red light increases the amount of both LHCP and SSU transcripts detected in nuclei harvested 2 hr later. The magnitude of this increase varies somewhat among experiments, depending on the levels remaining in the dark-treated plants. In the case of the SSU sequences, in which the amount remaining in the dark was substantial, the apparent increase is not as dramatic as that seen for the LHCP sequences. However, these increases represent minimal values (see below). The stimulatory effect of red light can always be reversed if far-red light

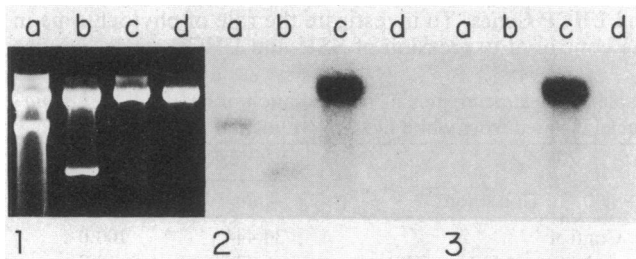


FIG. 3. Sensitivity of specific transcripts to α -amanitin. Southern blots of the following DNAs, cleaved with the indicated restriction endonuclease, were prepared: lanes a, pLgAB19/H5c and *Hind*III; lanes b, pLgSSU1 and *Pst* I; lanes c, pLg52 and *Hind*III; and lanes d, pBR322 and *Hind*III. Blots were hybridized with nuclear transcripts labeled with 32 P in either the presence or the absence of α -amanitin at 300 μ g/ml. 1, Gel stained with ethidium bromide; 2, control (no α -amanitin); 3, + α -amanitin at 300 μ g/ml. Hybrids were visualized by autoradiography.



FIG. 4. Hybridization of pLgSSU1 and pLgAB19/H5c to *in vitro* labeled nuclear transcripts. Plants were treated with four different light regimes, and Rc, D, R, and RFR nuclei were isolated. RNA was prepared from each incubation, and equal aliquots of 10^6 cpm were used to probe Southern blots of pLgSSU1 and pLgAB19/H5c. Hybrids were detected by autoradiography.

is immediately given. It is evident that reversal is complete for SSU but not LHCP sequences under these conditions. The far-red light reversal of the effect of red light on the labeling of these two transcripts demonstrates that phytochrome is involved in the control of their transcription or turnover.

SSU and LHCP Sequences Transcribed in Isolated Nuclei Are Not Rapidly Turned Over. To investigate the possibility that phytochrome acts to influence turnover or transcription itself, D nuclei were pulse labeled for 10 min and the amount of SSU and LHCP transcripts remaining was measured during a 110-min chase period (Table 2). It is clear that, while there is some loss of labeled transcripts during the 110-min chase, there is no substantial turnover during the 30-min incubation used sufficient to account for the difference between the amount of transcription in Rc and D nuclei. We conclude, therefore, that phytochrome acts to increase the transcription of RNA sequences encoding SSU and LHCP rather than to decrease turnover.

Effect of Light on rRNA Transcription. The relative amounts of rRNA synthesis, as judged by α -amanitin-resistant polymerase activity, differ in nuclei from plants grown under the different conditions of illumination. In the light-grown plants, about 40% of the radioactivity incorporated into RNA is resistant to α -amanitin (Table 1). In the plants grown in the dark with intermittent red illumination (Rc plants), 67% ($\pm 5\%$) of the radioactivity incorporated into RNA *in vitro* is resistant to α -amanitin at 300 μ g/ml. When these plants are placed in the dark for 7 days, the total radioactivity incorporated into RNA by the isolated nuclei decreases by 60–80% and the percentage of the radioactivity resistant to α -amanitin decreases to 31% ($\pm 1\%$). This suggests that red light also has an effect on rRNA transcription. Therefore, we have used the 25S rRNA-specific probe to show that transcription of rRNA sequences in *Lemna* nuclei is also affected by phytochrome (Fig. 5). A single minute of red light followed by 2 hr of darkness (R) is sufficient to result in 2-fold stimulation of 25S rRNA transcription compared to dark-treated plants (D). The effect of red light is reversible by immediate treatment with far-red light (RFR). The apparent increase in amount of rRNA transcription in the RFR sample as compared to D is similar to the amount stimulated by 10 min of far-red light alone followed by 2 hr of darkness (FR).

DISCUSSION

In this study we have characterized an isolated nuclear transcription system from *Lemna gibba*. Transcripts synthesized in this system are of substantial size (Fig. 2) as compared to some earlier studies (43, 44). Moreover, Southern hybridizations of restriction digests of SSU genomic clones (8) with *in vitro* labeled transcripts show that only certain specific sequences are transcribed from these large (44- to 48-kilobase) clones (unpublished data). We have been able to use this *in vitro* transcription system to show that phytochrome is in-

Table 2. Stability of SSU and LHCP sequences pulse labeled for 10 min with [32 P]UTP

Pulse, min	Chase, min	Input, cpm	cpm hybridized to filter*		% hybridization of input $\times 10^4$	
			pLgSSU	pLgAB 19/H5c	pLgSSU1	pLgAB 19/H5c
10	—	820,000	20.0	6.9	24.4	8.4
10	10	760,000	12.5	6.4	16.5	8.4
10	30	680,000	14.4	9.2	21.2	13.5
10	50	700,000	10.9	9.4	15.6	13.4
10	110	520,000	10.1	3.2	19.4	6.2

Linearized plasmid DNA bound to nitrocellulose (5 μ g per filter) was incubated with the appropriate radioactive probe for 3 days in 1 ml of hybridization buffer.

*Values are means of replicates, corrected for hybridization to pBR322 (mean value 1.7 cpm).

involved in the rapid regulation of the expression of two nucleus-encoded chloroplast protein genes by increasing their transcription relative to other transcripts.

Phytochrome is known to be involved in the regulation of expression of SSU, LHCP, and several other nucleus-encoded chloroplast proteins at the level of translatable mRNA. In the case of the NADPH-protochlorophyllide reductase (45, 46) and phytochrome itself (47, 48), phytochrome action is involved in the dark induction of mRNA. Phytochrome is also involved in the regulation of the RNA for the chloroplast DNA-encoded, 32,000 M_r thylakoid protein in mustard (49). There are a number of cases in which the white-light induction of translatable mRNAs could potentially involve phytochrome (19, 50–52). SSU transcription in pea (53) has been shown to be greater in light-grown than in dark-grown seedlings. The present study shows that transcripts labeled *in vitro* in isolated *Lemna* nuclei have the same relative amounts of SSU and LHCP sequences after the different light treatments as they do of these translatable (23) and hybridizable (24) mRNAs. It is consistent with the evidence that phytochrome mediates the light induction of expression of the SSU and LHCP genes by increasing their transcription.

The fact that red light also increases transcription of rRNA sequences actually masks a part of the increase in synthesis of the SSU and LHCP sequences in response to phytochrome action. Red light does not significantly increase the total radioactivity incorporated into RNA in isolated nuclei compared to the dark-treated control plants (unpublished data). Since a larger proportion of the total radioactivity incorporated after red light treatment is due to rRNA synthesis and because equal amounts of radioactivity are used in each hybridization, our estimates of the relative phytochrome-induced increases in the transcription of SSU and LHCP sequences are minimal values.

Another possibility which must be considered is that the green safelight may be sufficient to activate a very sensitive response (cf. ref. 54). If this is the case, the apparent magnitude of the response to red light would be reduced, and it would also suggest that the transcription of SSU and LHCP genes is differentially sensitive to red light. It is interesting

that in the case of the LHCP, reversal of the red light stimulus by far-red light appears incomplete in both this study and at the level of total mRNA (24). It is possible that the "escape time" for the light effect on the LHCP genes is less than 1 min, or that the phytochrome response is so sensitive that far-red light can act as a low-level stimulus in itself. The latter explanation seems more likely, since 10 min of far-red light alone results in some stimulation of transcription of pLg52 sequences (Fig. 5) and LHCP and SSU sequences (data not shown). Short escape times from phytochrome action have already been observed in another *Lemna* species, *L. perpusilla*, where induction of flowering can be inhibited by red light but not reversed by far-red light (55).

The mode of action of phytochrome is still unknown. However, this system offers the potential for studying how phytochrome may be linked to transcription. In addition to the rapid and marked effect of light on the expression of SSU and LHCP genes (24), there is the possibility of looking at the expression of individual members of each gene family. It seems unlikely that the phytochrome molecule itself acts to stimulate the transcription of SSU and LHCP sequences, since it is found primarily in the cytoplasm (56) and is known to rapidly affect nonnuclear events [e.g., chloroplast movement in *Mougeotia* (57)]. One possible mechanism is the involvement of intermediate factors induced or mobilized by phytochrome that act directly on transcription. Such a situation has already been shown to occur in prolactin induction of mRNA (58). The *Lemna* system offers an ideal opportunity to resolve the steps involved in phytochrome action on gene expression.

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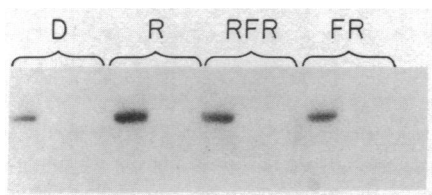


FIG. 5. Hybridization of pLg52 to *in vitro* labeled nuclear transcripts. Replicate Southern blots of pLg52 cleaved with *Hind*III (left lanes) and pBR322 cleaved with *Hind*III (right lanes) were hybridized to equal aliquots (0.5×10^6 cpm) of 32 P-labeled transcripts prepared from D, R, RFR, and FR nuclear incubations. Hybrids were detected by autoradiography.

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