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# Fast light-regulated genes of *Neurospora crassa*

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## ABSTRACT

Several physiological reactions including the sexual differentiation of the ascomycete *Neurospora crassa* are triggered by blue light. Mutants in the white-collar genes *wc*-1 and *wc*-2 are blind for all the blue light effects tested so far. We have previously shown that blue light induces some translatable mRNAs at different times after beginning the illumination. Here we report the cDNA cloning of four genes that are induced by blue light. Induction of these transcripts is temporally ordered (lag times from 2 to 45 min). Analysis of run-on transcripts show that the increases in mRNA levels are due to *de novo* transcription. None of these transcripts is inducible in white-collar mutants.

## INTRODUCTION

Light is one of the most important and best studied stimuli in higher plants and fungi. Different effects including differentiation and gene-expression are elicited by blue/UV-A (1, 2), by UV-B (2, 3) and by red/far-red light (2, 4). Generally the effect on gene-expression is at the level of transcription, but some examples of translational control are also known (5). The fastest light-regulated genes known until now are induced after 20 min by white light (6). In the ascomycete *Neurospora crassa* several effects are induced by blue light (7-9): Synthesis of carotenoids (10), induction of protoperithecia (11), hyperpolarisation of the membrane potential and change of input resistence (12), phototropism of the perithecial necks (13), regulation of the circadian rhythm of the *bd*-mutant (14, 15). The time between stimulus and reaction differs from 1 minute (change of input resistance (12)) to 24 hours (production of sexual structures called protoperithecia; (16)). The induction of protopherithecia is a differentiation process in which many genes will probably be involved. For an understanding of this differentiation process at the genetic level it is necessary to answer the following question: How many genes are regulated by blue light and in what way ? From *in vitro* translation data of isolated mRNA of *Neurospora crassa* we know that the first new translatable mRNA appears 2 minutes after the blue light signal, and that within the first 30 minutes there appear to be as many as 60-80 genes regulated. These genes can be classified in four induction groups. The lag times in the increase of the amount of mRNA are 2, 5, 10 and 20 min after the beginning of blue light illumination (Nawrath and Russo, Journal of Photochemistry and Photobiology,

 

in press). This means that the regulation is not only very fast but also time-staggered. The *wc*-mutants are blind for all physiological blue light effects tested so far (8, 15, 16). The enzymatic activities of the *al*-1, *al*-2 and *al*-3 gene products which are involved in the carotinoid synthesis are light regulated (16). Mutants in any of the three *al* genes have an albino phenotype. Recently the *al*-3 gene was cloned and it was shown that its mRNA is light regulated (17). The albino genes are not necessary for sexual morphogenesis (18). Therefore, for an understanding of photomorphogenesis, it was necessary to search for other light-regulated genes. Here we report the cDNA cloning of four blue light regulated genes. For two of those genes the amount of mRNA increases after 2 min, for the third gene after 15 min and for the last gene 45 min after induction. The *wc*-mutants are blind for this effect. Results from nuclear run-on analysis suggest that these effects are at least partly regulated at the level of transcription.

## MATERIALS AND METHODS

Strains:

*Neurospora crassa* strains: *wt* (STa), *wc*-1 (allele ER 53), *wc*-2 (allele ER 33) are isogenic and described in (19).

cDNA cloning:

Poly(A)$^+$ RNA from illuminated (60 min white light) *wt* mycelia of *Neurospora crassa* was converted to double stranded cDNA with the Amersham cDNA synthesis kit. From 5 $\mu$g of mRNA approximately 1 $\mu$g of cDNA was prepared. The average length of the single stranded products was about 1300 nucleotides as estimated by alkaline agarose gel electrophoresis. The cDNA was then *Eco*RI methylated and, after addition of *Eco*RI linkers, cloned into the *Eco*RI site of *kgt*10. The bacterial host was *E. coli* C600. Approximately 8x $10^5$ lambda recombinants were generated in this way.

The $\lambda gt10$ bank was differentially screened by using labelled cDNA. This was done at low density (500 plaques per petri dish) using duplicate pairs of filters, two hybridized with control cDNA made from dark grown cultures and two with cDNA made from illuminated cultures. Hybridization was done according to (20).

Illumination of mycelia:

Illumination was carried out as described (9). The fluence rate of the blue light was 14 W/m$^2$ and that of the red light 78 W/m$^2$. The fluence rate of the blue part of the white light was 6 W/m$^2$. In the blue light series the illumination time was for a maximum of 10 min. The time of extraction given in fig. 1 is the time from the beginning of the illumination. In the white light series the illumination was continuous for 60 min and then the mycelia were extracted immediately. For each time point we used the same culture for the illuminated and dark control mycelium. This was achieved by cutting the mycelial mat into two halves; one half was illuminated and the other was used as the dark control.

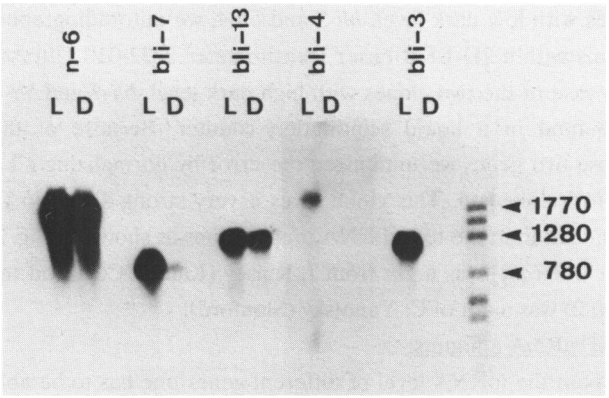


Figure 1: Northern blots
2 µg of poly(A)$^+$ RNA were loaded on each lane. An RNA molecular weight marker (BRL) was run in parallel. The length is indicated in nucleotides. The probe in each Northern blot was the $^{32}$P labelled cDNA fragment. L: poly(A)$^+$ RNA from 60 min white light treated mycelium; D: poly(A)$^+$ RNA from the corresponding dark mycelium.

Northern blot:

Northern blotting was done on Nylon membranes (GeneScreen, Du Pont) as recommended by the suppliers.

Dot blot:

For RNA extraction see (21). For RNA dot blots see (22). We used different concentrations of RNA as indicated in fig 2. For radiolabelling of cDNA fragments see (23). The quantification of the hybridized filter was done in two different ways. In the case

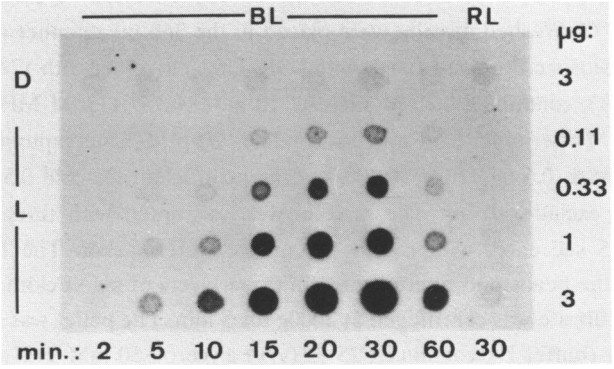


Figure 2: Dot blot of *bli*-4
D: total RNA isolated from a dark grown culture, L: total RNA isolated from an illuminated culture, BL: blue light, RL: red light, µg: amount of total RNA in the dot.

of the two clones with low dark level, *bli*-3 and *bli*-4, we autoradiographed the filters and analysed the films with a 2D LKB Laser Densitometer 2222-010 Ultrascan XL and LKB software. In the case of the two clones with high dark level, *bli*-7 and *bli*-13, the dots were cut out and counted in a liquid scintillation counter. Because of the smaller light induction of those two genes we minimized the error by normalizing all RNA extractions against the control clone *n*-6. This clone gives a very strong signal in the dot blots and shows no light induction in the tested RNA preparations as shown in Fig. 3c.The glutamate dehydrogenase clone (*am*) was a gift from J. Kinsey (Kansas City) and the clone of the ß-tubulin gene (*tub* 2) was a gift of C. Yanofsky (Stanford).

Quantification of mRNA amounts:

In order to compare the mRNA level of different genes one has to be able to quantify the mRNA amounts for each clone. Theoretically the mRNA amount in a given dot can be calculated from the amount of radioactive cDNA bound to the mRNA in that dot. For this purpose one needs a calibration curve obtained by a series of dots with different amounts of pure mRNA of the gene analysed. However we do not have pure mRNA from the *bli* genes. We assumed therefore that RNA/DNA hybridization was as efficient as DNA/DNA hybridization and we made the calibration curve for each clone making a series of dots with different amounts of cDNA. We are aware that our assumption is not completely correct (24) but the inherent error should only influence the absolute value of mRNA and not the relative values of the different mRNA amounts from our clones.

Isolation of nuclei:

Nuclei were isolated by a modification of the method of Willmitzer and Wagner (25). *Neurospora* mycelia (18-20 g fresh weight) were harvested and photo-induced (either for 30 or for 60 min white light) as described previously (9). After light or dark incubation, mycelia were washed once in ice-cold water. All subsequent steps were carried out as close as possible to 0°C. Washed mycelia were placed in the 360 ml chamber of a Bead Beater cell disrupter (Biospec Products, Bartlesville, OK, USA) together with 170 ml of extraction buffer (buffer A), containing 0.25 M sucrose, 10 mM NaCl, 10 mM MES pH 6.0, 5 mM EDTA, 0.15 mM spermine, 0.50 mM spermidine, 20 mM 2-mercaptoethanol, 0.20 mM PMSF, 0.1 % BSA, 0.6 % Triton X 100, and then sufficient ice-cold 0.5 mm glass beads were added to exclude all air. The mycelia were disrupted with three 30 sec bursts, separated by 45 sec each, with cooling by an ice/water jacket. The homogenate was decanted from the beads and filtered through three layers of nylon cloth (120, 80, and 20 µm mesh). The filtrate was centrifuged at 2000g for 5 min. The pellet was resuspended in 5 ml nuclei buffer (buffer B), containing 25 % (v/v) glycerol, 50 mM Tris acetate pH 7.8, 5 mM $Mg(OAc)_2$, 0.10 mM EDTA, 5mM DTT, 0.15 mM spermine, 0.50 mM spermidine, 20 µM Pepstatin A, 1 % Trasylol, 1 mM EGTA, 0.1 mM PMSF, 0.1 % BSA, and centrifuged at 2000g for 5 min. The resulting pellet was resuspended in 5 ml buffer B with Pepstatin A

and PMSF omitted (buffer C), and centrifuged at 1000g for 5 min. The final pellet was resuspended in 500 µl of buffer C with BSA omitted and stored at -20°C.

Run-on transcription:

A standard transcription assay (400 µl) contained 200 µl nuclear suspension and 200 µl transcription mixture, 180 mM KOAc, 20 mM $Mg(OAc)_2$, 1 mM ATP, 1 mM CTP, 1 mM UTP, 50 µM S-adenosylmethionine and 200-500 µCi of [$\alpha$-$^{32}$P]-GTP (410 Ci/mmol). Transcription was started by addition of the nuclear suspension and the assay mixture was incubated at 25°C for 5 min. Termination of transcription and RNA isolation were performed according to (26).

Plasmid DNA (5 µg) was linearized by restriction enzyme digestion. The DNA was denatured and attached to nylon membranes as described (22). After autoradiography the optical density in each spot was measured with a 2D LKB Laser Densitometer. The ratio between the transcription efficiency of nuclei extracted from illuminated and dark control mycelia of the clone *n*-6 was defined as 1. All the other values were normalized against clone *n*-6.

## RESULTS

cDNA cloning of blue light regulated genes.

For the cloning of the genes we constructed a cDNA library in $\lambda gt10$ with mRNA isolated from cultures illuminated with white light for 60 min. The library was differentially screened with radioactively labelled cDNA made from mRNA isolated from light treated (60 min white light) and dark control liquid cultures. We obtained 4 clones which are light regulated. As shown by cross-hybridization analysis, these are different from each other and from *al*-1 and *al*-2 (which have been cloned by Schmidhauser and Yanofsky, data not shown). The size of the cDNA inserts in the four clones *bli*-3, *bli*-4, *bli*-7 and *bli*-13 was 860, 740, 750 and 900 basepairs, respectively (data not shown). A Northern blot analysis showed that the mRNAs have lengths between 1000 and 1900 nucleotides (fig.1). The four light regulated genes each hybridizes to a single transcript. The control clone *n*-6 seems to be expressed in two different mRNA species. Two very close bands can be seen on the autoradiogram after short time exposure (data not shown). The Northern blot (fig.1) shows that all four genes are clearly light induced.

Characterization of blue light regulated genes.

For further characterization (and especially to see how fast these 4 genes are regulated) it was necessary to determine the kinetics of the mRNA increase. To get an induction profile we performed RNA dot blots with total RNA. The cultures for RNA isolation were irradiated with blue light for a maximum of 10 min. RNA was extracted at different times after the beginning of illumination. The data in Fig. 3 show that the genes *bli*-3 and *bli*-4 have a very similar induction profile. The increase of the mRNA level started after 2 min,

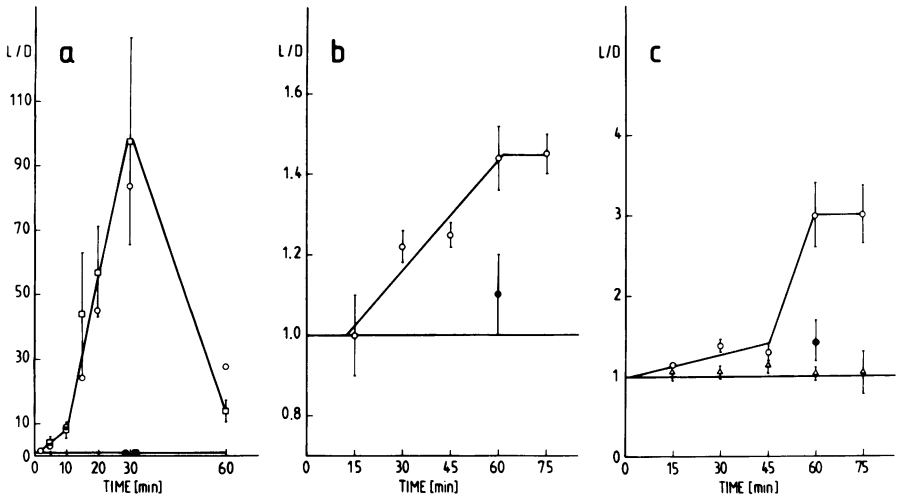


Figure 3: Kinetics of mRNA levels of the four *bli* genes after blue light illumination.
For each time point we extracted RNA from illuminated and from dark control cultures. The given values L/D are the ratios between the amount of specific RNA in the blue light treated mycelium and the amount of specific RNA in the dark mycelium. The kinetics of *bli*-3, *bli*-4 and *n*-6 correspond to values obtained directly from the quantification of the dot blots. The values of the kinetics of *bli*-7 and *bli*-13 were first normalized to *n*-6.
The red light control is shown as a full symbol. Each point is the average of three to five RNA dot blots with independent total RNA preparations.
a) Kinetics of *bli*-3 (open circles), and *bli*-4 (squares)
b) kinetics of *bli*-13
c) kinetics of *bli*-7 (open circles), and *n*-6 (triangles)

and has a maximum at 30 min with a 90-fold increase over the dark level in both cases. The expression of these two genes in the dark is quite low. From the profile we estimated the half-lives of the mRNAs to be at most 15 min.

The genes *bli*-7 and *bli*-13 behave differently. They are already expressed quite strongly in the dark, and their relative induction by blue light is much lower (3-fold for *bli*-7 and 50 % for *bli*-13). Both are more strongly induced by continuous illumination of 60 min with white light (table 1). They have a longer lag time of induction , 15 min for *bli*-13 and 45 min for *bli*-7, and their profile does not show a maximum as do *bli*-3 and *bli*-4, but rather a plateau between 60 min and 75 min. After induction the amount of mRNA in all four cloned genes is much higher than the amount of the mRNA for glutamate dehydrogenase. This gene, and a second non-regulated clone *n*-6 which we isolated, were included in the analysis as controls and reference genes.

The dark value of the amount of *bli*-7 mRNA was very variable in different extracts. The reason for this is not yet known. The dark amounts of the different mRNAs are shown in

**Table 1: Characterization of the light-regulated genes.**

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|
| genes | mRNA length | lag time | L/D *WT* | | L/D wc-1 WL | L/D wc-2 WL | ng/mg total RNA | run-on Exp. L/D *WT* | |
| | | | BL | WL | | | | 30 min | 60 min |
| *bli*-3 | 1100 | 2 | 84 | 65 (12) | 0.6 (0.05) | 1.2 (0.3) | 0.4 | 15/10.3 | -/1.5/1.9 |
| *bli*-4 | 1900 | 2 | 95 | 59 (13) | 0.9 (0.1) | 1.1 (0.2) | 0.1 | 3.6/6.8 | 1.6/-/1.3 |
| *bli*-7 | 1000 | 45 | 3 | 16 (4) | 1.5 (0.2) | 1.2 | 6-50 | 1.2/1.6 | 1.7/2.3/1.6 |
| *bli*-13 | 1300 | 15 | 1.45 | 1.8 (0.2) | 0.9 (0.04) | 1.1 (0.1) | 10 | 0.8/1.2 | 1.3/1.4/1.4 |
| *n*-6 | 1700 1900 | -- | 1.04 | 1.3 (0.08) | 1.1 (0.2) | 1.2 (0.1) | 55 | 1 / 1 | 1 / 1 / 1 |
| *am* | | -- | 0.9 (0.1) | 0.8 (0.1) | 0.6 (0.1) | 0.9 (0.2) | 1.6 | 1.3/1.6 | 0.8/0.9/1.1 |

The table summarizes all data of the four light regulated genes and the non-regulated controls (*n6*, *am*). Length of the corresponding mRNAs (column 2) is given in nucleotides (see Fig. 1). Lag times in the mRNA increase (column 3), are given in minutes. The given values L/D are the ratios between the amount of specific RNA in the blue light treated mycelium and the amount of specific RNA in the dark mycelium. L/D *wt* for the blue light series (BL) (column 4) is the maximal value from Fig. 2. To compare the light induction of the *wc* mutants ( *wc*-1, *wc*-2 ) with that of *wt* we performed RNA dot blots with total RNA isolated from *wc* cultures illuminated 60 min. with white light (WL) (column 5,6 and 7). The SEM of three to five independent experiments is given in parentheses. The amount of specific RNA in the total RNA of dark mycelia is given in column 8, the SEM was less than 30%. The results of the run-on analysis are given in columns 9 and 10. The transcriptional activation after 30 min white light is given in column 9, while values after 60 min white light are shown in column 10. The data of the run-on are values from independent experiments.

column 8 of Table 1. In this table a summary of all results of the four *bli* genes and control genes is given.

Gene regulation in the wc mutants.

The *wc*-genes are necessary for all physiological effects triggered by blue light. Therefore it is important to examine the expression of the cloned genes in these mutants. Dot blots with RNA isolated from *wc*-1 and *wc*-2 mycelia illuminated with white light for 60 min showed that all four *bli* genes were not induced in the two pleiotropic mutants. They were only expressed at the basal level of the *wt* in the dark (table 1). This means that the products of the *wc*-genes are necessary for the regulation of the *bli* genes by blue light.

Run-on transcription of the cloned genes.

In the RNA dot blots we observed an increased steady state level of specific RNAs. With this experiment it is not possible to distinguish between increased transcriptional initiation or increased stability of the mRNA. To answer this question we performed run-on transcription experiments with isolated nuclei from illuminated and dark control cultures. We used continuous illumination with white light, because under those conditions *bli*-7 and *bli*-13 were maximally induced. The data in Table 1 (column 9) show a significant induction of initiation of transcription for *bli*-3 and *bli*-4 after 30 min white light illumination. After 60 min illumination the induction of transcription is much less than at 30 min. In the case of *bli*-7 and *bli*-13 the situation is reversed; there is only a small induction of transcription (if any) after 30 min, whereas it is higher after 60 min. The genes *am* and *tub*-2 used as controls were not light regulated (fig. 4).

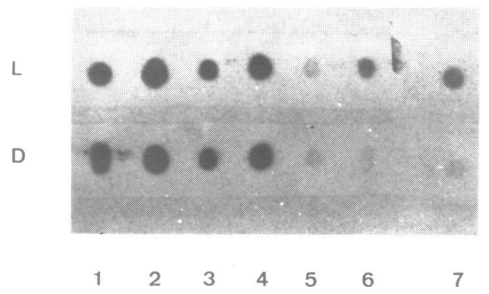


Figure 4: Run-on experiments
Labelled RNA synthesized by nuclei isolated from dark grown (D) and light illuminated (L) (30 min white light) *Neurospora crassa* mycelia were hybridized to plasmid DNAs immobilized to the filter (1: *n*-6, 2: *am*, 3: *tub*-2, 4: *bli*-13, 5: *bli*-7, 6: *bli*-4, 7: *bli*-3).
The same amount of radioactivity ($10^7$ cpm) was used for each hybridization, and the extent of hybridization visualized by autoradiography. X-ray films were exposed for an identical period of time. The quantitative analysis of the dots is given in table 1.

## DISCUSSION

We have cloned cDNAs from four genes which are light regulated. We named these genes *bli* (blue light induced). The mRNA profile after induction by blue light shows that the induction occurs at different times after illumination. The *bli*-3 and *bli*-4 genes are induced very rapidly (2 min lag time), whereas *bli*-13 is induced after 15 min and *bli*-7 after 45 min. The level of induction is very high for the early light regulated genes (*bli*-3 and *bli*-4) while it is relatively low for the late regulated genes (*bli*-7 and *bli*-13). The dark values of mRNA are also very different between early and late regulated genes. The early regulated genes have a dark value of mRNA of about $10^{-3}$ % of the poly(A)$^+$ mRNA, whereas the late regulated genes have a value of about 0.1 %, which is a difference of a factor of 100. These values are calculated from the data in table 1, assuming that poly(A)$^+$ RNA in *Neurospora* comprises about 1 % of the total RNA (27). Because of the great differences in the dark values of mRNA it is not too surprising that the late regulated genes have a lower light induction as compared to the two early regulated genes. The run-on experiments from 30 min illumination show clearly that, at least for the *bli*-3 and *bli*-4 genes, the increased level of mRNA is due to an increased transcription rate. On the other hand after 60 min continuous illumination the rate of transcription is close to the dark value, while the steady state level of mRNA is very high compared to the dark value. This could mean that there is a light effect on the stability of the specific mRNAs at a late time after illumination. For *bli*-7 and *bli*-13 the situation is completely different. The rate of transcription at 60 min after illumination is higher than at 30 min, as we would expect for late regulated genes.

According to the characterization of these genes none of them coincides with the *al*-3 gene cloned recently (17). As reported under "results" we showed by cross-hybridi-zation experiments that they do not coincide with *al*-1 and *al*-2. It remains to be shown whether the *bli* genes are necessary for photomorphogenesis.

The *bli*-3 and *bii*-4 genes are the fastest light regulated genes known until now. If we take into account that there is probably not enough time between the light stimulus and the appearence of *bli*-3 and *bli*-4 to produce a regulatory factor (28), it is very likely that the hypothetical regulating factor is present in the cell before light treatment. This could be an activator protein that is only able to bind to the promoters of the genes after a light induced modification. It is interesting to note that a protein dephosphorylation was found in *Neurospora crassa* very early after light induction (Lauter and Russo, manuscript in preparation).

The mutants in the *wc*-1 and *wc*-2 genes block all the known photoeffects. None of the four *bli* genes were induced in these mutants. As assumed by Degli Innocenti and Russo (8) the *wc*-genes could code for the photoreceptor or for the first part of the sensory transduction

chain. This means that one of the *wc*-genes could also code for the positive acting factor proposed above.

The time dependent transcription of the genes could be explained either by a cascade regulation where fast regulated genes (like *bli*-3 and *bli*-4) are necessary for late light regulated genes (like *bli*-7 and *bli*-13), or by different affinities of the promotor elements for one or a few blue light transcription factors (4).

The further cloning of the genomic copies and sequencing of the genes should help to find the elements which are important for this transcriptional regulation by blue light. These four cDNA clones begin to help our understanding of the molecular biology of blue light induced differentiation in *Neurospora crassa.*

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