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Measurement of *in Vivo* DNA Binding by Sequence-Specific Transcription Factors Using UV Cross-Linking

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This paper describes an *in vivo* UV cross-linking protocol that is sensitive enough to detect DNA binding by sequence-specific transcription factors in *Drosophila* embryos and tissue culture cells. The strength of this approach is that it provides a quantitative measure of DNA binding *in vivo* with unambiguous identification of the factor involved in the binding. This assay often detects DNA binding properties of proteins that were not predicted from previous experiments, and it can be used to directly test diverse models of gene regulation in the context of a living organism.
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Over a decade of research has made it clear that the regulation of transcription in eukaryotic cells is extremely complex, involving input from many sequence-specific and general transcription factors as well as from chromatin structure (1, 2). To understand this complex phenomenon at the biochemical level, one approach has been to develop increasingly sophisticated *in vitro* transcription and DNA binding assays (3–6). A complementary approach has been to directly measure biochemical properties of regulatory molecules (usually DNA binding) in the context of the cell.

Many of these *in vivo* methods are footprinting assays (see articles in the first section of this issue). These assays give a high-resolution picture of the regions in a promoter that are occupied by DNA binding proteins *in vivo.* A drawback of these procedures is that they do not unambiguously identify the factor causing the footprint. A different class of *in vivo* DNA binding assays employs cross-linking of proteins to DNA *in vivo* and subsequent purification and characterization of the protein-DNA complexes (7–9; see also articles by Paro and co-workers (9a) and by Moss, Dimitrov, and Houde (9b) in this issue). Although these approaches generally lack the high resolution of *in vivo* footprinting, they have other advantages. First, they unambiguously identify the factor involved in binding since they generally employ an immunoprecipitation step. This can be critical in many cases, for example, when it is necessary to distinguish between members of a family of transcription factors that bind to the same sequences. In addition, *in vivo* cross-linking can more conveniently survey binding to large regions of DNA, and unlike *in vivo* footprinting, very low levels of binding can be detected.

In the *in vivo* UV cross-linking method of Gilmour *et al.* (7), proteins that have been cross-linked to DNA *in vivo* with UV light are immunoprecipitated, and the attached DNAs are characterized by Southern blotting. A particular strength of this method is that UV light induces covalent bonds only between species that are in intimate contact with another, eliminating the possibility of nonspecific cross-linking (10). This method has been used to study the DNA binding of several proteins in *Drosophila* tissue culture cells and embryos (11–14). For example, UV cross-linking yielded the first evidence that there is a paused polymerase associated with the hsp70 promoter (12), an observation that was later confirmed by other methods (15).

Recently, we have developed an *in vivo* UV cross-linking protocol (16) that is considerably more sensitive than the original procedure of Gilmour and Lis (12). The more sensitive protocol makes it possible to detect *in vivo* DNA binding by sequence-specific transcription factors despite the fact that they cross-link to DNA with relatively low efficiency. In other experiments, we have demonstrated that for many transcription factors, UV cross-linking yields a highly accurate measure of DNA binding (17). Therefore, UV cross-linking can now be used to assess, in a quantitative manner, the DNA

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binding of sequence-specific transcription factors *in vivo.* This technique has been used to measure the DNA binding of several transcription factors to a wide range of gene fragments in *Drosophila* embryos and tissue culture cells (16, 18). The results of these experiments have provided fresh perspectives on how transcription factors function in the context of a cell (see Concluding Remarks). Below, we discuss the *in vivo* UV cross-linking technique for use on *Drosophila* embryos and tissue culture cells and the potential application to other organisms.

PRELIMINARY EXPERIMENTS

Does the Protein of Interest Cross-Link to DNA?

A prerequisite for studying any protein–DNA interaction by UV cross-linking is that the protein must cross-link to DNA with reasonable efficiency. A likely requirement for efficient cross-linking is that the binding site of the protein must contain thymidine residues as these cross-link to protein far more efficiently than the other nucleotides (10). However, a precise geometry between protein and nucleic acid residues is also required for efficient cross-linking to occur (19), as the presence of thymidine residues in the recognition sequence does not guarantee efficient cross-linking. For these reasons, it is advisable to examine the efficiency of UV cross-linking of a protein to a typical binding site *in vitro* before attempting to observe cross-linking *in vivo.*

A very simple *in vitro* assay can be used to determine the efficiency of cross-linking. The purified DNA binding protein is incubated with about 10–20 fmol of an oligonucleotide (15–30 residues long) that has been radioactively labeled and that contains a specific binding site. It is very important that the binding reaction contain 0.05% NP-40 or an equivalent detergent because this prevents loss of the protein upon UV irradiation. The binding reaction is spotted on a parafilm-covered cold metal block at a distance of 3 cm from the bulbs and is UV-irradiated for 1–4 min using the lamp described below for *in vivo* UV cross-linking. The reaction is then mixed immediately with SDS sample buffer and separated on a SDS–polyacrylamide gel. Oligonucleotides with protein covalently attached migrate more slowly in the gel than the free oligonucleotide and appear as a shifted species (see Fig. 1). Assuming that all the oligonucleotide was bound by protein before irradiation, the percentage of shifted oligonucleotide represents the cross-linking efficiency. A value of 0.2–1% is typical for sequence-specific transcription factors whose binding has successfully been examined *in vivo* by UV cross-linking.

Is Cross-Linking Proportional to DNA Binding?

Recent experiments show that the relative levels of UV cross-linking and DNA binding of sequence-specific transcription factors is proportional on a wide range of DNA fragments (17; J. Walter and M. D. Biggin, unpublished results). For example, this proportionality is observed with the *eve* protein (see Fig. 2) and may be due to the fact that homeodomain proteins such as *eve* bind to sites that contain an ATTA core so that cross-linking to all sites is similarly efficient. The proportionality between binding and cross-linking also applies to the *zeste* transcription factor (data not shown). However, it is possible that for some proteins this proportionality may not apply, especially in cases where small numbers of binding sites are examined. Therefore, we recommend that an *in vitro* cross-linking experiment like the one shown in Fig. 2 be used to determine whether cross-linking and binding are proportional on the same sequences that will be examined *in vivo.* This control is extremely important if the *in vivo* cross-linking results are to be interpreted quantitatively.

To perform this control, a binding reaction such as the one described in the last section is irradiated as described above. Instead of an oligonucleotide, how-

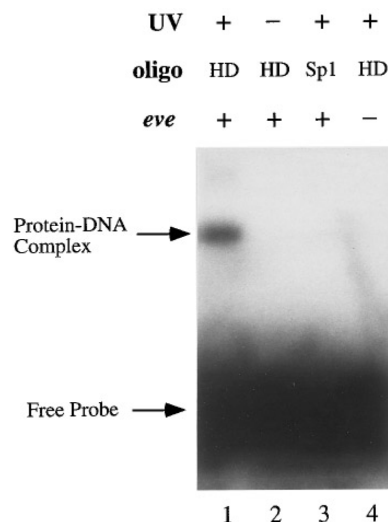

FIG. 1. UV cross-linking of *eve* protein to an oligonucleotide. To give the result shown in lane 1, 60 ng of bacterially expressed *eve* protein (21) was incubated in 32.5 mM Hepes, pH 7.6, 0.05 mM EDTA, 6.25 mM $MgCl_2$, 100 mM KCl, 5% glycerol, 0.05% NP-40, 50 μg/ml poly(dI-dC), and 13 fmol of radiolabeled FPB oligonucleotide (22) in a total volume of 20 μl for 30 min on ice. The mixture was then UV irradiated and separated on a 10% SDS polyacrylamide gel as described in the text (Preliminary Experiments). The gel was dried onto Whatman filter paper and subjected to autoradiography. In lane 2, the reaction was not UV irradiated; in lane 3, binding was examined to an oligonucleotide which contains an Sp1 binding site (23); in lane 4 *eve* protein was omitted from the reaction. Quantitation shows that *eve* protein is covalently bound to about 1% of the input oligonucleotide.

ever, the binding reaction should contain a cocktail of radiolabeled DNA fragments containing the sequences that will be examined by *in vivo* UV cross-linking. The irradiated reaction is added to 200 μl of chromatin restriction buffer (for composition of buffers, see below), which disrupts noncovalent interactions. A primary antibody against the protein under study is added to the mixture and incubated for 1.5 h at 4°C before the addition of 10 μl of 20% staph A cells. After a 30-min incubation, staph A cells are collected by centrifugation and washed twice in dialysis buffer containing 0.2% Sarkosyl and twice in immunoprecipitation buffer. The DNA is eluted by vortexing the cells in the presence of 100 μl of elution buffer for 10 min (this step can be repeated if all the radioactivity is not released from the cells). After the DNA is eluted, 60 μl of proteinase K dilution buffer containing 1 mg/ml freshly added proteinase K is added, and the mixture is incubated for 30 min at 60°C. The mixture is then extracted once with phenol/chloroform, extracted once with chloroform, ethanol precipitated in the presence of 40 μg carrier RNA, and analyzed by electrophoresis and autoradiography. For comparison, binding in the same reaction is also compared using a standard assay such as filter binding, and the results of the two types of assays are compared (Fig. 2).

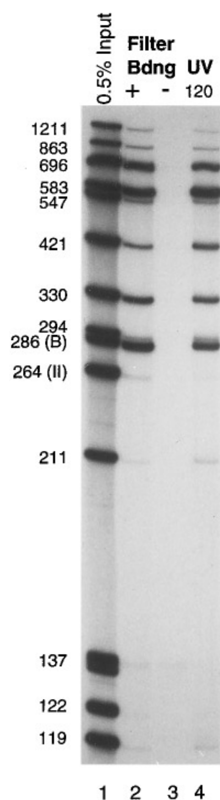

FIG. 2. Cross-linking by *eve* protein closely parallels the level of binding to many DNA fragments (taken from Ref. 17). Binding of *eve* protein to a restriction digest of a 3.5-kb *Eco*RI *Ubx* proximal promoter fragment was examined using filter binding (lanes 2 and 3) or *in vitro* UV cross-linking (lane 4). Binding reactions with 400 ng of *eve* protein and 10 fmol of each DNA fragment were set up as described in the legend to Fig. 1. Filter binding (lanes 2 and 3) was carried out as described previously (24); Reaction 4 was irradiated for 2 min as described in the legend to Fig. 1 after which it was mixed with 200 μl of chromatin restriction buffer. 4.5 μg of purified anti-*eve* antibody (16) was added for 1.5 h at 4°C before the addition of 10 μl of 20% staph A cells. After 30 min of incubation, staph A cells were collected by centrifugation and processed as described in the text to isolate the cross-linked DNA. 1.6% of filter bound products (lanes 2 and 3), all of the UV cross-linked products (lane 4), and 0.5% of the starting DNA (lane 1) were separated on a 6% sequencing gel.

IN VIVO UV CROSS-LINKING OF SEQUENCE-SPECIFIC TRANSCRIPTION FACTORS

There are several important considerations when using *in vivo* cross-linking to study binding in *Drosophila* embryos. First, a sizable quantity of embryos (0.5–2 g) is required for a single experiment. Therefore, if the strain used is not highly robust, it can be impractical to collect such large amounts of embryos. Second, the protein under examination should be expressed in at least 10% of the cells in the embryo to achieve reasonable cross-linking signals. Finally, the observed cross-linking efficiency is an average of all the cells expressing the protein. Therefore, it is best to study regulatory interactions that are equivalent in most cells of the embryo. Finally, we note that the cross-linking efficiency achieved *in vivo* is considerably lower than that achieved *in vitro* (compare Figs. 3 and 2), probably because UV exposure is greatly attenuated in living tissue.

UV Irradiation of *Drosophila* Embryos

Using standard techniques, embryos are collected from population cages that are maintained at 24.5°C and 60% humidity and that contain 60–100 ml of adult flies. Embryos are removed from the cages, aged for the desired amount of time, and then isolated and weighed. To increase UV exposure, the embryos are dechorionated in fresh 50% Chlorox bleach for 2 min.

All subsequent manipulations are carried out at 4°C. The dechorionated embryos are suspended in 40 ml of cold 0.1% Tween 20 by vortexing at high speed for 5–10 s and distributed among two plastic trays for irradiation. The equivalent of 5 g of undechorionated embryos can be irradiated at a single time. The trays are elevated to be at a distance of 3 cm from the light bulbs of a Fotodyne DNA Transfer Lamp, which emits 254 nm light from four 15-W bulbs. The trays are cooled from below by an ice-water bath. The two trays are chosen so that together they match the dimensions of the four bulbs, which cover an area of about 72 square inches. Embryos are irradiated for six 5-min time peri-

ods. Between irradiations, the trays are rocked to reorient the embryos. It is not recommended to irradiate embryos for longer than 30 min because chromatin becomes degraded, insoluble, and impossible to digest with restriction enzymes. After irradiation, embryos are either immediately processed to extract chromatin or frozen in liquid nitrogen where they are stable for at least several months.

Chromatin Extraction and Purification

Table 1 can be consulted to determine what size rotor and how many gradients should be used to purify chromatin from a given quantity of embryos. Five to ten grams of embryos are dounced in 35 ml of NIB buffer using a Teflon pestle at 5000 rpm for one stroke to disrupt all aggregates and then dounced for two additional strokes at 4000 rpm. The homogenate is passed through prewetted miracloth (Calbiochem) into a prechilled beaker containing a stir bar. While stirring briskly, 20% Triton X-100 is added to a final concentration of 0.3%. The suspension is distributed among Sorvall SS34 tubes and centrifuged for 15 min at 4000 rpm and 4°C in an SS34 rotor. Supernatants are aspirated off starting at the top of the tube to ensure removal of lipids.

Cold nuclei lysis buffer is added to each tube. The volume of buffer is calculated from the amount of starting embryos and the number of CsCl step gradients to be used (see Table 1). Nuclei pellets are resuspended, transferred to a manual B dounce, and completely homogenized. Then 0.1 vol of 20% sarkosyl is added with vigorous mixing, and each sample is sheared by making two passages through an 18-gauge needle and two additional passages through a 25-gauge needle. Passage through the 25-gauge needle is most easily done by pouring the mixture into the top of the syringe and pushing it out through the needle. The sheared chromatin is kept on ice while the CsCl step gradients are poured (see Table 1). Note that if the gradients are overloaded with too much extract, degradation of the chromatin can occur.

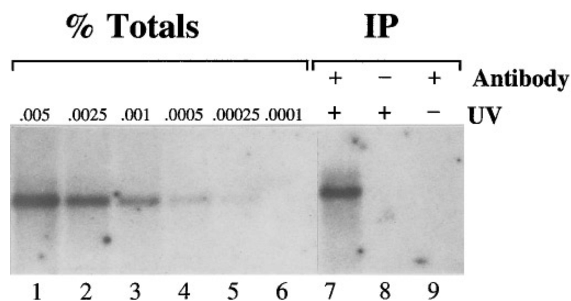

FIG. 3. *eve* protein binds to the *eve* promoter in embryos (Adapted from 16). Southern blotting shows the results of an *in vivo* UV cross-linking experiment carried out on irradiated 4- to 5-h-old embryos (lanes 7 and 8) or unirradiated 4- to 5-h-old embryos (lane 9) according to the procedure described in the text. Chromatin from embryos was isolated, digested with *Bgl*I, and 450 μg of chromatin was immunoprecipitated with affinity-purified *eve* antibodies (lanes 7 and 9) or mock-precipitated (lane 8). The immunoprecipitated DNA was analyzed on a Southern blot using *eve* promoter sequences as a probe detecting a 7.3-kb *eve* promoter fragment extending from −0.3 to −7.6 kb. For quantitation of the immunoprecipitated material, lanes 1–6 contain a known percentage of the DNA present in a single immunoprecipitation reaction prior to the addition of antibody (referred to as %Total DNA). For further controls demonstrating the specificity of the cross-linking signal, see Ref. 16.

To harvest SW28 gradients, an 18-gauge needle is inserted 1 cm below the discrete milky white band (located about one-third of the way from the bottom of the tube) and twelve 1.0-ml fractions are collected slowly. For SW41 gradients, the 18-gauge needle is inserted 0.7 cm below the white band and ten 0.25-ml fractions are collected.

One microliter of each fraction is analyzed by electrophoresis in a 0.7% agarose gel containing 0.3 μg/ml of ethidium bromide. Fractions containing high-molecular-weight chromatin are pooled. If any peak fractions contain significant amounts of a white precipitate, these should be filtered through a 5-μm syringe filter. The pooled fractions are dialyzed using spectrapor spec2 tubing for 3× 2 h against 1.5 liters of dialysis buffer. We have modified the previous composition for the dialysis buffer (7) by removing the sarkosyl. Using the sarkosyl-free buffer does not reduce recovery of protein–DNA products but dramatically increases the ability of certain restriction enzymes (such as *Pst*I) to digest the chromatin.

We have recently discovered that *in vivo* cross-linking of some proteins is difficult to detect due to proteolysis (Alan Carr and M. D. Biggin, unpublished results). This problem can be avoided if all solutions that come into contact with the cross-linked chromatin during purification and immunoprecipitation are supplemented with PMSF to 1 mM just before use (dialysis buffer can be supplemented to 0.5 mM). Also, solutions should be filter-sterilized and stored at 4°C in clean containers.

After dialysis, the chromatin is centrifuged for 10 min at 500*g* at room temperature to remove any insoluble material. The conductivity of the dialyzed chromatin should be measured to make sure that dialysis is complete. The approximate concentration of the chromatin can be determined by measuring the absorbance at 260 nm. Approximate chromatin yields per gram of irradiated embryos are 200 μg for 4- to 5-h embryos, 500 μg for 5- to 7-h embryos, and 800 μg for 8- to 10-h embryos. The purified chromatin is frozen in liquid nitrogen in aliquots and stored at −80°C. Chromatin should be thawed in a 4°C water bath, and it can be frozen and thawed several times.

Chromatin Digestion

To detect DNA binding by most transcription factors in *Drosophila* embryos, 450 μg of digested chromatin

should be used in a single immunoprecipitation. Four hundred fifty micrograms of chromatin is typically digested in a volume of 1.5–1.7 ml. In addition to the chromatin, the digest should contain the restriction buffer recommended by the supplier of the enzyme, RIA grade BSA (Sigma) at a final concentration of 100 μg/ml, and Triton X-100 at a final concentration of 0.01%. This mixture is incubated at 37°C for 10 min before adding the restriction enzyme. Typically, 0.25 units of enzyme per 1 μg of DNA is used in a 12-h digest. A second identical addition of enzyme is made at least 4 h after starting the digest. To make sure that the enzyme(s) have cut efficiently, 2 μl of the digest is analyzed on a 0.7% agarose gel.

Gilmour *et al.* (7) discuss which enzymes cut chromatin efficiently. As noted above, some enzymes that did not cut under the original conditions of Gilmour and Lis (12) now cut the chromatin very efficiently. Enzymes that require low-salt buffers may now also work. Furthermore, double and triple digests with enzymes that cut efficiently can be conveniently carried out.

For the last hour of the digest, ~60 μg of DNAse free RNAse A is added to reactions containing 450 μg of DNA. After RNase A digestion, reactions are stopped with 1/25 volume of 0.5 M EDTA, pH 7.9, and supplemented with Triton X-100 and sarkosyl to final concentrations of 0.3 and 0.05%, respectively. Samples are centrifuged for 10 min at 500–1000*g* to remove insoluble material and then transferred to fresh tubes.

Immunoprecipitation (IP)

The primary antibody is incubated with the digested chromatin for 3 h at 4°C with continuous rocking. Generally, 0.5–2 μg of an affinity-purified antibody should be used in a 1.5-ml reaction containing 450 μg of chromatin. Using affinity-purified antibody reduces the possibility of cross-reaction with other proteins and allows one to use a minimum of staph A cells, which can cause background. In some cases, crude sera with very high titers have also worked (J. Walter and M. D. Biggin, unpublished results). After the primary antibody has been added, preparation of staph A cells should be initiated (see next section). For primary antibodies whose Fc portion is not efficiently bound by staph A cells (such as goat IgG), an appropriate secondary antibody should be added and incubated with the chromatin for 1 h at 4°C while rocking.

After the primary and secondary antibody incubations, samples are spun for 15 min at top speed in a microcentrifuge at 4°C. The supernatant of each reaction is filtered separately through a fresh 0.2-μm syringe filter into a fresh tube (Millipore filters are preferable although GelmanSciences Sterile Acrodiscs are also adequate). The centrifugation and filtration steps are critical to remove nonspecifically aggregated chromatin.

To precipitate immune complexes, 25 μl of a 20% suspension of staph A cells (see next section) are added to each IP and the mixture is incubated for 15 min at room temperature while rocking. Staph A cells are collected by spinning the reaction for 1 min at top speed in a microfuge. One percent of the supernatant from this spin is removed. This so-called "Total DNA" (7) is processed in parallel with the immunoprecipitated DNA once it has been eluted from the staph A cells. When loaded on the Southern blotting gel alongside

TABLE 1

Parameters for Cross-linking Experiments

Embryos			
4–5 h	ND	3–6 g	15–23 g
8–10 h	ND	1–3 g	5–7.5 g
Number of cells: (for tissue culture experiments)	$<3 \times 10^8$	3×10^8 to 5×10^8	2×10^9
Nuclear lysis			
Nuclear lysis buffer	0.9 ml	2.7 ml	8.1 ml
20% Sarkosyl	0.1 ml	0.3 ml	0.9 ml
Ultracentrifugation			
Rotor size (Beckman)	SW60	SW41	SW28
CsCl steps			
1.75 g/ml	1.3 ml	4.5 ml	18.5 ml
1.5 g/ml	0.9 ml	2.3 ml	6.0 ml
1.3 g/ml	0.7 ml	1.5 ml	3.5 ml
Run speed/time: (20°C)	30K/~20 h	37K/~24 h	26K/~40 h
CsCl preparation			
Density	g CsCl	ml Buffer[a]	Refractive index
1.75 g/ml	100	75	1.404
1.50 g/ml	66.7	83.3	1.381
1.30 g/ml	40.0	90	1.363

Note. Based on Refs. (7, 13).
[a] Buffer should contain final concentrations of 0.5% Sarkosyl, 1 mM EDTA.

the precipitated DNA, the total DNA serves as a standard by which the efficiency of the IP is judged.

The staph A pellets are washed twice in dialysis buffer containing 0.2% sarkosyl at room temperature and then four times in immunoprecipitation buffer. To wash the staph A pellets, they are first resuspended in 200 μl of buffer using a pipetteman, and the pipette tip is washed once with 200 μl of fresh buffer to avoid loss of staph A cells. Before centrifugation, another 1 ml of buffer is added. During the last wash, pellets are transferred to a new set of tubes to reduce background.

To elute DNA from the staph A cells, the staph A pellets are resuspended in 100 μl of elution buffer and vortexed on a multitube vortexer at medium speed for 10 min. Cells are pelleted by centrifugation as usual and the supernatant is transferred to clean tubes. This elution procedure is repeated two more times. To the 300 μl of eluted DNA, 200 μl of proteinase K dilution buffer is added. At this point the total DNA sample is digested with proteinase K exactly as the eluted DNA. The proteinase K digestion must proceed for at least 10 h at 60°C. (On rare occasions, it has been necessary to extract the eluted DNA with phenol/chloroform and chloroform after the proteinase K digest to prevent anomalous migration of the DNA on the Southern blot.)

After digestion with proteinase K, the DNA is ethanol precipitated by the addition of 50 μl of 3 M NaAc, pH 5.3, 40 μg of carrier RNA, and 1.25 ml of ethanol. The mixture is chilled at −20°C for 1 h and then at −70°C for 30 min. Once thawed, the sample is spun for 15 min at 4°C, the clearly visible white pellets are washed in 75% ethanol and vacuum dried. Pellets are resuspended in 20 μl of loading dye for electrophoresis on an agarose gel.

Preparation of Staph A Cells

It is important to use staph A cells from Boehringer Mannheim because those from other suppliers have given highly variable results. The cells are prepared according to the following procedure to reduce nonspecific sticking of the chromatin to the staph A cells. Protein A agarose and other forms of coupled protein A do not work in this assay.

To make a stock solution of staph A cells, 10–40 ml of 10% staph A cells are washed twice in 1 vol of dialysis buffer containing 0.2% Sarkosyl and then resuspended in 2 vol of PBS, 3% SDS, and 10% betamercaptoethanol. The cells are boiled for 30 min after which they are washed twice in dialysis buffer containing 0.2% Sarkosyl and resuspended in the same buffer as a 20% suspension. To avoid freeze–thawing, cells are frozen as 100-μl aliquots in liquid nitrogen and stored at −70°C at which temperature they are stable for at least 1 year.

On the day of the experiment, following the addition of the primary antibody, staph A cells are thawed and washed once with dialysis buffer. The cells are then resuspended in 2 vol of blocking chromatin. Blocking chromatin is prepared by sonicating dialyzed and irradiated chromatin from 8- to 10-h-old embryos to an average length of about 1 kb. Sonicated chromatin from irradiated tissue culture cells should also be an adequate source of blocking chromatin. The mixture of sonicated chromatin and staph A cells is rocked for 2–3 h at 4°C. The cells are then washed once in dialysis buffer containing 0.2% sarkosyl (when using monoclonal antibodies, it may be important to leave out sarkosyl) and resuspended in dialysis buffer containing 0.2% sarkosyl to give a 20% suspension that is added to the immunoprecipitation reaction.

Southern Blotting

The following highly sensitive Southern blotting protocol has been designed for Amersham Hybond-N (uncharged nylon) membranes. Very high concentrations of probe are used to achieve maximal signals, and strict adherence to the protocol is necessary to avoid high filter backgrounds. Using this protocol, one can conveniently detect 100 fg of complementary plasmid DNA overnight and as little as 10 fg in a 5-day exposure. Similarly, a 0.005% cross-linking signal from 450 μg of *Drosophila* chromatin should be clearly visible overnight and very strong after a 1-week exposure (see Fig. 3).

Immunoprecipitated and total DNA samples are separated on a 0.7% agarose gel in 1× TBE running buffer lacking ethidium bromide. After electrophoresis, the gel is stained for 5 min in 1.5 μg/ml ethidium bromide, 1× TBE and then destained for 10 min in 1× TBE. The gel should be photographed with UV light to verify that recovery of DNA was efficient (the 300 ng of carrier DNA derived from the elution buffer should be clearly visible as a smear).

The gel is incubated in denaturation buffer for 30 min with gentle rocking, rinsed in water, and incubated for two 15-min periods in a generous amount of neutralization buffer with gentle rocking. The DNA is transferred to presoaked Hybond-N membrane in 20× SSPE for at least 10 h. After transfer, the nylon membrane is incubated in 2× SSPE for 5 min and then gently blotted dry on filter paper. The membrane is dried for exactly 30 min at 80°C in a vacuum oven and then it is irradiated with UV light using the same DNA transfer lamp as above. The optimal irradiation time (5–30 s) must be determined empirically for every batch of membrane. After irradiation, the membrane is briefly wetted in double distilled water, placed in prehybridization solution, and incubated at 42°C for at least 16 h with shaking.

For the prehybridization, plastic Tupperware boxes with sealed tops and very smooth, flat bottoms are used, and about 0.2 ml of prehybridization solution per cm^2 of the bottom of the container is used. It is essential

that blots be completely covered in liquid at all times. Incubating two blots in the same container causes high filter background.

For greatest sensitivity, 16×10^6 cpm/ml of a probe (and no more) with specific activity of about 5×10^9 cpm/μg is used in the hybridization. Unincorporated label should be completely removed from the probe as this causes high filter background. After denaturing and snap-cooling the finished probe, it should be centrifuged for 2–5 min in a microfuge at 4°C to remove insoluble material, which causes filter backgrounds. The blot is lifted out of the solution with forceps while the probe is mixed into the prehybridization solution, and the blot is lowered back into the solution. Hybridization is allowed to proceed for 18–24 h at 42°C with brisk shaking. Membranes are washed two times for 15 min each in 2× SSPE, 0.1% SDS at 25°C, then for 15 min in 1× SSPE, 0.1% SDS at 65°C, and finally for 60 min in 0.1× SSPE, 1.0% SDS at 65°C. After the last wash, if the background is greater than 2 cps, one can continue washing the blot in fresh 0.1× SSPE, 1.0% SDS for up to 12 h at 65°C.

In Vivo UV Cross-Linking Using *Drosophila* Tissue Culture Cells

Cross-linking on tissue culture cells has several advantages, and in many cases it may be necessary to study a protein–DNA interaction in this system before attempting experiments in an intact organism. First, it is easier to achieve high levels of UV exposure in isolated cells. Second, through transfection of expression and reporter plasmids, the levels of the DNA binding protein and the target DNA can be raised to high levels. This may be essential to observe cross-linking in cases where cross-linking is inefficient or when the genome of the organism is very large. Finally, a cell line can often be found that does not express the protein of interest. Thus, by transfecting these cells with appropriate expression and control plasmids, the dependence of any UV cross-linking on the presence of the transcription factor can be rigorously examined.

In principle, many different cell lines and transfection methods should give good results with UV cross-linking. When using the calcium-phosphate transfection method on *Drosophila* tissue culture cells, the following protocol has given good results (16). After transfection of cells with appropriate amounts of expression and reporter plasmids (16), they are typically maintained for 1 or 2 days to allow the transcription factor to accumulate to high levels. Subsequently, cells are mixed with 1/500 vol of 12% HCl to dissolve calcium phosphate precipitates, pelleted, washed in 0.7 vol of cell wash buffer, pelleted again, and resuspended in 1 vol of cold PBS. If untransfected cells are used in the procedure, they are simply washed and resuspended in PBS. Cells (2.4×10^8) are irradiated using the same arrangement as for embryos except that irradiation is for 4 times 1 min with gentle shaking between irradiation periods. It is important to note that overexposure to UV light will cause degradation of the DNA and difficulties in digesting the DNA with restriction enzymes. After irradiation, the cells are spun down, resuspended in nuclei lysis buffer, and lysed with 1/10 vol of 20% sarkosyl (see Table 1). The chromatin is sheared by five passages through a 25-gauge needle and loaded onto a CsCl step gradient (see Table 1). Each immunoprecipitation is carried out with chromatin from about 4×10^7 cells exactly as described for DNA isolated from embryos (see above).

CONTROL EXPERIMENTS

If at first one cannot detect DNA binding by a transcription factor *in vivo* using UV cross-linking, there are several controls that can be carried out. As mentioned above, it should be verified that the transcription factor can be cross-linked to DNA *in vitro.* If this is the case, it suggests that cross-linked species made *in vivo* are not being efficiently isolated. To identify problematic steps in the procedure, protein–DNA adducts should be made *in vitro* as described above using large fragments of radiolabeled DNA, and these should be added to the *in vivo* cross-linking protocol at different stages. If the radioactive cross-linked species are lost in the CsCl purification, it may suggest that the protein is being degraded by proteases. Alternatively, if the adducts are lost during the immunoprecipitation, it suggests that the parameters of this reaction must be adjusted. It is important to note that, preformed staph A–antibody complexes precipitate covalent protein–DNA complexes very inefficiently, whereas sequential addition of antibody and staph A cells gives much higher yields of covalent complexes.

APPLICATION OF IN VIVO UV CROSS-LINKING TO OTHER ORGANISMS

It should be possible to carry out *in vivo* UV cross-linking on other organisms. Several issues should be taken into consideration: Is there an abundant source of tissue that can be conveniently UV irradiated? In the case of *eve* protein, chromatin from about 10^7 cells is required to observe strong cross-linking signals. How large is the genome of the organism? For organisms with smaller genome sizes, the amount of chromatin in 10^7 cells will be less, making the experiment easier. In the case of organisms with larger genome sizes, more chromatin will be required. For example, in mamma-

lian cells, 20 times more chromatin would be required to obtain the same cross-linking signal as in *Drosophila* cells if all other parameters were equal. We suggest that when carrying out UV cross-linking on mammalian cells, cross-linking should first be examined to a plasmid that has been transfected into tissue culture cells in many copies (see above). Once a signal has been obtained in this system, it could be attempted to observe cross-linking to an endogenous locus.

If *in vivo* UV cross-linking is carried out on non-*Drosophila* cells, the optimum amount of UV exposure should be determined anew. The optimum can be found by irradiating cells for increasing amounts of time and then examining the chromatin for physical degradation and the extent to which it can be digested with restriction enzymes such as *Eco*RI. The optimum time is when inhibition of *Eco*RI is no greater than 10% and before significant degradation of the DNA is observed. Finally, the number of cells loaded onto a CsCl gradient should be corrected for the genome size of the organism to avoid overloading or underloading of gradients.

CONCLUDING REMARKS

In vivo cross-linking has been used for several years to study protein–DNA interactions in *Drosophila* cells. The *in vivo* UV cross-linking protocol described here now makes it possible to examine *in vivo* DNA binding of sequence-specific transcription factors. This method appears to be generally applicable since it has been utilized to study DNA binding by five different transcription factors containing four distinct DNA binding domains (16, 18; J. Laney and M. D. Biggin, unpublished results). The method is versatile in other regards since it can be used to study DNA binding to endogenous genes as well as to mutated transgenic promoter constructs (16a). Other parameters such as the developmental stage at which cross-linking is examined can be conveniently varied. However, due to the large amounts of tissue required in this protocol, it is currently not possible to carry out cross-linking on embryos carrying lethal mutations or mutations that reduce the viability or fertility of flies (16), preventing an important class of experiment in many cases.

A similar approach is the *in vivo* formaldehyde cross-linking method, which uses formaldehyde instead of UV light to induce bonds between protein and DNA *in vivo* (see article in this issue by R. Paro and co-workers (9a)). An advantage of this approach is that it is much more efficient than UV cross-linking, allowing one to work with smaller quantities of tissue. However, it is not clear to what extent formaldehyde cross-linking is a quantitative measure of DNA binding. Also, proteins that do not bind directly to DNA may be cross-linked to DNA indirectly in this method.

The quantitative and highly specific nature of the *in vivo* UV cross-linking assay has made it possible to distinguish between competing models of how certain transcription factors function *in vivo.* For example, it has been controversial whether related homeodomain proteins bind to the same or different sequences in *Drosophila* embryos. Strongly supporting the latter view, the relative levels of *in vivo* UV cross-linking of two homeodomain proteins *even-skipped* (*eve*) and *fushi-tarazu* (*ftz*) are very similar on a large range of DNA fragments (16). In this case, the fact that the UV cross-linking protocol can distinguish binding by highly related transcription factors was critical. In another example, *in vivo* UV cross-linking has helped to elucidate the nature of the redundant regulation of the *Ultrabithorax* gene by the *zeste* transcription factor (16a). Cross-linking of *zeste* protein to the *Ubx* promoter in embryos supports a model in which different redundant transcriptional mechanisms regulating the *Ubx* gene act simultaneously rather than being organized as primary and back-up systems. This example illustrates the usefulness of the assay for studying phenomena such as transcriptional redundancy that do not have easily scorable phenotypes.

The procedure described here has also successfully uncovered DNA binding properties of transcription factors that were not predicted from other indirect assays. For example, it was discovered that *eve* and *ftz* proteins bind at apparently uniform levels to many kilobase-pairs of DNA throughout certain promoters, raising the possibility that homeodomain proteins regulate transcription by entirely novel mechanisms (16). In another example, it was shown that the GAGA transcription factor binds at high levels to the body of the *hsp70* gene upon heat shock induction, suggesting that this protein may play an active role in rendering the *hsp70* gene accessible to RNA polymerase II (18). Together with the earlier results on RNA polymerase and other DNA binding proteins (12, 20), these results illustrate the power of *in vivo* UV cross-linking for studying regulatory interactions in the organism.

SOLUTIONS AND BUFFERS

NIB. 0.3 M sucrose, 15 mM NaCl, 5 mM $MgCl_2$, 15 mM Tris pH 7.5, 60 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA.

Nuclei lysis buffer. 100 mM NaCl, 10 mM Tris, pH 8, 1 mM EDTA, 0.1% NP-40, 0.5 mM phenylmethylsulfonyl fluoride.

Dialysis buffer. 2 mM EDTA, 50 mM Tris, pH 8.0.

Immunoprecipitation buffer. 100 mM Tris, pH 9.0, 500 mM LiCl, 1% NP-40, 1% deoxycholic acid.

Elution buffer. 50 mM $NaHCO_3$, 1% SDS, 1.5 μg/ml sonicated calf thymus DNA; pH is adjusted to 10.0 with NaOH. Since the carrier DNA can show up as a smear on the Southern blot it should be sonicated to a small size (about 1 kb) that will not overlap with most restriction fragments.

Proteinase K dilution buffer. 0.3% SDS, 50 mM Tris 7.5, 10 mM EDTA, 1 mg/ml Proteinase K (added fresh as solid).

Cell wash buffer. 137 mM NaCl, 2.7 mM KCl, 5 mM BisTris, pH 6.0.

20× SSPE. 3.6 M NaCl, 0.2 M $NaPO_4$-monobasic, 20 mM EDTA, adjust pH to 8.0 and filter through 0.22-μm filter.

Denaturation solution. 1.5 M NaCl, 0.5 M NaOH.

Neutralization buffer. 1.5 M NaCl, 0.5 M Tris, 1 mM EDTA, adjust pH to 7.2.

Prehybridization solution. This solution should contain the final concentrations listed below. Ingredients are added in the order listed (starting with the formamide) while stirring vigorously on a warm stir plate. When all solids have dissolved, the solution is filtered through a 0.45-μm syringe filter. It may be necessary to change the filter after about 30 ml due to clogging. Use the solution immediately.

50% formamide; use 100% Fluka–Chemika.
6× SSPE; use a 0.22-μm filtered 20× stock.
90 μg/ml sonicated calf thymus DNA with average length ca. 1 kb; make a 1 mg/ml stock in TE, sonicate, denature half of it by boiling, recombine the two aliquots, and freeze the DNA at −20°C in 5- to 10-ml aliquots. If half of this DNA is not properly denatured, hybridization of the probe to nonspecific DNA sequences can occur.
10 μg/ml *Escherichia coli* strain K genomic DNA (Sigma); use a 1 mg/ml stock in TE which has been sheared through a 25-gauge needle several times and which is stored at −20°C.
5× Denhardt's solution; use a 50× filter-sterilized stock which is kept at −20°C as 5- to 10-ml aliquots.
10% dextran sulfate; add solid from Pharmacia.
5% SDS: add solid.
1% Sarkosyl: add solid.

Chromatin restriction buffer. 150 mM NaCl, 10 mM Tris pH 7.5, 10 mM $MgCl_2$, 1% Triton X-100, 0.2% Sarkosyl, and 100 μg/ml RIA grade BSA (Sigma), 0.2 mg/ml sonicated calf thymus DNA.

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