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A *Drosophila Adh* gene can be activated in *trans* by an enhancer

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ABSTRACT

The ability of a segment of the *Drosophila Adh* gene to produce ADH activity in larvae is dependent upon the presence of a 53 bp sequence (called NS1) located between 289 and 341 bp upstream of the larval transcription start site. This sequence behaves like an enhancer in that it can stimulate gene activity when it is placed at various distances from, or on either side of, an *Adh* gene. Like a typical enhancer, NS1 does not ordinarily function in *trans*. However, when an *Adh* gene lacking NS1 is placed on one plasmid, and a second gene carrying NS1 is placed on another, and the two plasmids are interlocked in a catenane, both genes are active. This finding supports the mechanism of loop-mediated enhancer action.

INTRODUCTION

Enhancers are a class of *cis*-acting genetic elements that stimulate transcription (1–7). A number of models have been advanced in an effort to account for the ability of enhancers to act over great distances and in either orientation with respect to a structural gene. Two appear particularly promising. The first is a scanning model, where the enhancer binds one or more transcription factors at a distant site and the resultant transcription complex slides along the DNA to the site of transcription initiation. The second is a looping model, where the enhancer and transcription initiation regions are brought into proximity by proteins bound to both sites while the intervening DNA forms a loop (2, 5, 6, 8–11).

Our interest in enhancers and their mechanism of action stems from our analysis of the control of expression of the *Adh* gene of *Drosophila melanogaster*. We have been studying the regulation of *Adh* using a technique that we call somatic transformation (12–14). In this procedure, we injected plasmids containing the *Adh* gene into early embryos and assessed the activity of the gene in third instar larvae of the injected generation. One of our initial findings was that the activity of the *Hpa*I to *Xba*I fragment of the gene—a construct containing 400 bp upstream of the proximal start site—was eliminated when a 53 bp region (−289 to −341; the NS1 region) of DNA was deleted. Activity could be restored by the presence of a second gene with an intact NS1 region on the same plasmid (13), or by the presence of multiple copies of a portion of the NS1 region on the same plasmid (M. Yablonsky and W. Sofer, unpublished observations). Activity could not be restored to the deleted gene by the coinjection of a second plasmid containing an intact gene (14). Thus the NS1 region has many of the properties of an enhancer element.

In this report, we ask whether the rescue of an NS1-deleted gene's activity only occurs when an intact gene is on the same molecule (consistent with the scanning model) or whether rescue can also occur when the two genes are on separate plasmids that are in close proximity (consistent with the looping model). We show here that the activity of an NS1-deleted gene can be rescued in *trans* by an intact gene if the two genes are on separate plasmids that are interlocked with one another. These results provide support for the looping mechanism of enhancer action.

MATERIALS AND METHODS

Enzymes

Phage lambda integrase and *Escherichia coli* integration host factor (IHF) were generously provided by H. N. Nash. All other enzymes were purchased from New England Biolabs or Bethesda Research Laboratories.

Plasmid constructs

Manipulations were carried out using standard techniques (15, 16). The plasmid (pWX3501; Figure 1a) used in this work to generate the catenane (Figure 1b) was a construct containing an intact Adh^F gene, an Adh^S gene harboring the NS1 deletion (13), and phage lambda *attP* and *attB* recombination sites (17, 18). (Two different *Adh* genes were utilized because they produce enzymes with different electrophoretic mobility, and it is enzyme activity that we monitor in these experiments.) It was assembled from a plasmid bearing an intact Adh^F gene and an NS1-deleted Adh^S gene (13) by introducing an *attB* site by ligation of an oligonucleotide after digestion of the plasmid with *Sph*I and *Sal*I. The oligonucleotide had the following sequence:

TCGACGTTGAAGCCTGCTTTTTTATACTAACTTGAGCGAAACGGCCGCATG
GCAACTTCGGACGAAAAAATATGATTGAACTCGCTTTGCCGGC

The *attP* site was added by the following procedure. Plasmid

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pHN894 (similar to pYK100 (19, 20), and a gift of Howard Nash) was cut with *Hinc*II, a 1200 bp fragment was isolated after agarose electrophoresis, *Eco*RI linkers were added, and the fragment was treated with *Eco*RI. This piece was ligated into the *attB*-containing plasmid described above after it had been linearized with *Eco*RI.

In vitro recombination reaction

The standard reaction conditions used 20 mM Tris-HCl (pH 7.5), 50 mM NaCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, 10 μg of DNA, 875 ng of integrase protein, and 3.0 μg of IHF protein in 600 μl of reaction solution. The reaction was allowed to proceed at 25°C for two hours. It was stopped by raising the temperature to 65°C for 10 minutes.

To show that the catenane had been produced and to evaluate the proportion of catenane formed, preparations were digested with *Eco*RI. Digestion of non-recombined material (pWX3501) produced fragments of 7006 and 803 bp (Figure 2, lane 3). On the other hand, digestion of the catenane with *Eco*RI linearized the two catenane rings and produced fragments of 5122 bp (carrying the Adh^F gene) and 2687 bp (carrying the NS1-deleted Adh^S gene) (Figure 2, lane 2). In a variety of experiments, the yield of catenane ranged from 25%−85%.

Isolation of pure catenane (nicking and chromatography)

Bovine serum albumin, ethidium bromide and DNase I were added to 600 μl of the recombination solution described above to a final concentration of 300 μg/ml, 345 μg/ml and 2.75 μg/ml respectively. The reaction was allowed to proceed at 30°C for 30 minutes, at which time it was cooled in ice and 25 μl of 0.5 M EDTA added. The reaction mixture was reduced to a suitable volume by either extraction with sec-butanol or evaporation at reduced pressure and loaded on a 20×25×0.7 cm gel containing 0.8% agarose and 0.03% SDS. Electrophoresis was carried out in TAE buffer containing 0.03% SDS for 3−4 days at 1 V/cm with recirculation of the buffer. After electrophoresis, the bands were located by staining with ethidium bromide, excised, placed in dialysis bags with TAE, and electroeluted at 35V overnight. The solution containing the DNA was concentrated with sec-butanol or by evaporation, 3 M sodium acetate was added, and the DNA precipitated with ethanol. The catenane was passed through a spin column equilibrated with 5 mM KCl and 0.10 mM Na_3PO_4 (pH 6.8) and then injected into *D.melanogaster*.

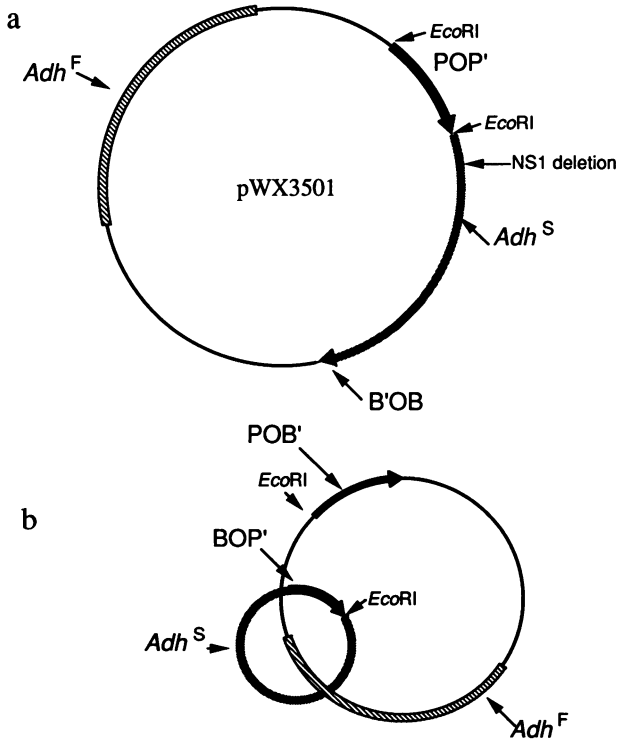

Figure 1. Plasmid pWX3501 before and after *in vitro* recombination. Some of the important features and restriction sites are shown. The figures are somewhat simplified in that both the catenane and pWX3501 are supercoiled, and the catenane consists of a mixture of overlays. (a) pWX3501. B'OB is the *attB* site and POP' is the *attP* site (17, 18). (b) Catenane produced by treatment of pWX3501 with integrase and IHF. BOP' and POB' represent recombination products.

Assay

D.melanogaster embryos of the Adh^{fn6} strain (21) which display no ADH enzymatic activity were somatically transformed with various concentrations of catenane using the microinjection technique described previously (12, 13). Third instar larvae of the injected generation were assayed for ADH^F and ADH^S activity by electrophoresing the extract from 1−4 homogenized larvae on cellulose acetate gels and subsequent staining (13) or on polyacrylamide gels using the Phast system (Pharmacia LKB Biotechnology Inc.). The ratio of activity of ADH^F and ADH^S was assessed by densitometry. Since the specific activity of Adh^S is approximately one-third that of Adh^F (22), densitometric values of the homodimers and heterodimer (recorded in arbitrary units) were normalized prior to the calculation of activity ratios.

Southern analysis

We used the procedure in Table 3 of Jowett (23) to extract DNA from 23−29 third instar larvae. Blotting was done on to Nytran nylon membranes using standard procedures.

RESULTS

Rescue

In the first experiment, an 85% pure preparation of catenanes carrying an NS1-deleted Adh^S gene on one ring and an intact Adh^F gene on the other was injected at 25 μg/ml. In all extracts tested we were able to detect ADH^F and ADH^S activity, as well as the heterodimer formed by the random combination of ADH^S and ADH^F subunits (Table 1, row 1), indicating that both genes were active. The ratio of activity detected was similar to that found when the unrecombined plasmid (pWX3501) carrying both genes was injected and analyzed (Table 1). The fact that an 85% pure preparation of the experimental catenane could support ADH^S activity in a ratio close to that of the two genes on a single plasmid was taken to indicate that an intact Adh^F gene can restore activity in *trans* to a gene lacking an enhancer when they are interlocked in a catenane.

We confirmed that the results were not due to the 15% contamination of the catenane with unrecombined plasmid in two ways. First, we injected unrecombined plasmid into embryos at the concentration of the contaminant (5 μg/ml). No ADH activity was found in 32 individuals assayed indicating that this concentration was too low to allow expression of detectable amounts of enzyme (larvae express ADH activity proportionally with respect to injected DNA (data not shown)). Second, we separated catenanes from unrecombined parental plasmid by

nicking with DNase I in the presence of ethidium bromide (24) and high resolution agarose gel electrophoresis (25). (We were unable to to separate supercoiled catenanes from parental plasmid.) We obtained material containing approximately 97% catenane relative to unrecombined plasmid (Figure 2, lane 2). When this material was injected at 38 μg/ml, 11 of 16 preparations (groups of four larvae homogenized together) exhibited detectable ADH activity (Table 1, row 2). In all cases, when activity was detected, both ADH^S and ADH^F forms were present. Another preparation of nicked material was injected at 125 μg/ml and single larvae homogenized. After electrophoresis (Figure 3), we detected ADH activity in 25 of 27 individuals tested (Table 1, row 3) and in all 25 cases ADH^S activity was restored. S/F activity ratios ranged from 0.2 to 0.8. Further, the Actual/Predicted ratios indicate that the amount of heterodimer formed was that expected from random association of the two types of monomers. These data show that both genes were active in the same cells at about the same time.

Table 1. ADH expression from catenane and unrecombined plasmid. These data represent the average of n electrophoretic determinations, with between 1 and 4 transformed larvae homogenized for each assay. The supercoiled catenane preparation was 85% pure. The nicked preparations were approximately 97% pure and injected at either 38 μg/ml or 125 μg/ml

Construct	ADH^S/ADH^F ± SD	Actual/Predicted[a] Heterodimer ± SD
Catenane (supercoiled; 25 μg/ml) n = 9	0.99 ± 0.12	1.00 ± 0.08
Catenane (nicked; 38 μg/ml) n = 11	0.88 ± 0.23	1.05 ± 0.19
Catenane (nicked; 125 μg/ml) n = 25	0.48 ± 0.18	1.33 ± 0.29
pWX3501 (unrecombined; 250 μg/ml) n = 7	1.02 ± 0.07	1.14 ± 0.11

[a]Predicted on the basis of random association of monomers

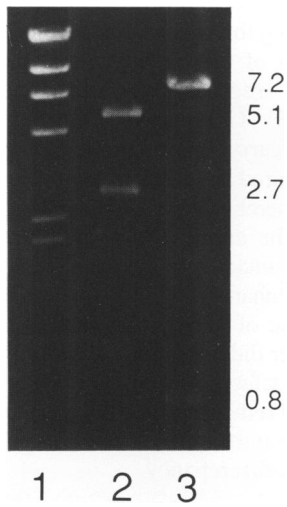

Figure 2. Assessing purity of catenane preparations. Photographs of ethidium bromide stained agarose gels after electrophoresis of DNA. Lane 1: Phage λ DNA digested with *Hin*dIII (size markers). Lane 2: Purified catenane (97% purity) digested with *Eco*RI. The linearized rings migrate to 5.1 and 2.7 kb. Lane 3: pWX3501 (unrecombined plasmid), digested with *Eco*RI, migrates to 7.2 and 0.8 kb.

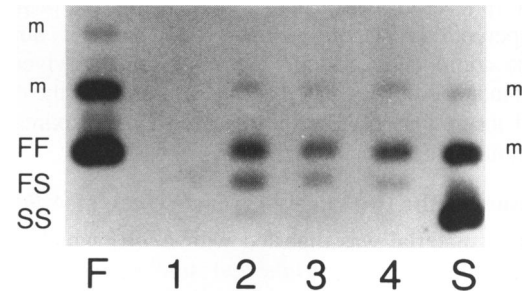

Figure 3. ADH enzyme activity assayed after injection of nicked catenane (97% pure). Lanes 1 through 4: Extracts from four individual third instar larvae injected as embryos with nicked catenane (125 μg/ml) were electrophoresed on a cellulose acetate membrane and stained for activity. The larva in lane 1 showed no detectable activity. Lanes labeled F and S are ADH^F and ADH^S standards. FF, SS and FS indicate the position of the major forms of the two homodimers and the heterodimer. The lowercase 'm' in the left and right margins indicates the locations of two minor forms of the allozymes. When both allozymes are electrophoresed in a single lane, one of the ADH^S minor forms overlaps the major form of ADH^F. The value of the minor form averages 7% of the densitometric value of the ADH^S major form and is subtracted from the value of the ADH^F major form. The specific activity of ADH^S is approximately one-third that of ADH^F (22), consequently, densitometric values (in arbitrary units) are normalized to obtain relative activities. ADH^S/ADH^F ratios were 0.4, 0.6 and 0.4 for lanes 2 , 3 and 4 respectively. The presence of S/F heterodimer indicates that both ADH^F and ADH^S monomers are being expressed in the same cells.

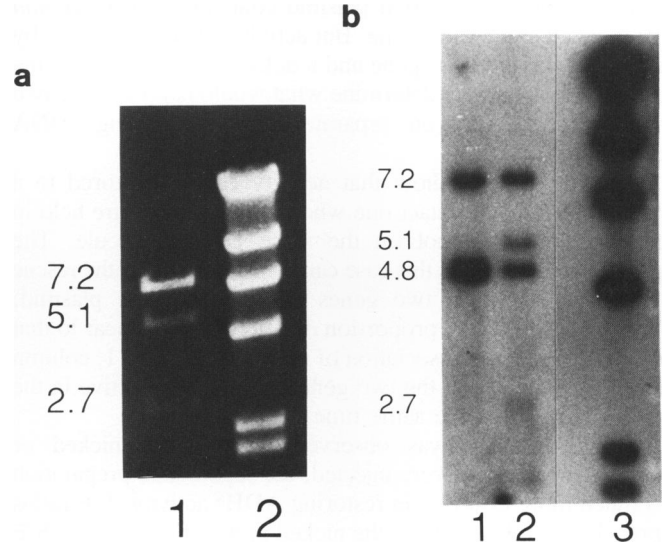

Figure 4. Survival of supercoiled pWX3501 and catenane after injection. (a) An ethidium bromide stained agarose gel. Lane 1: An *Eco*RI digestion of a 2:1 mixture of pWX3501 and catenane, prior to injection. Lane 2: Phage λ DNA size standard digested with *Hin*dIII. (b) Southern blot of DNA extracted from third instar larvae. The labelled probe is a 2.2 kb *Hpa*I to *Xba*I fragment. Lane 1: *Eco*RI digestion of pWX3501 injected without catenane (the 7.2 kb band is shown, the 0.8 kb band is not). The 4.8 kb band is the *Eco*RI fragment of the endogenous *Adh* gene (Adh^{fn6}). Lane 2: *Eco*RI digestion of pWX3501 (7.2 kb) and catenane (5.1 and 2.7 kb) injected as a mixture (2:1). Lane 3: Phage λ DNA size standard digested with *Hin*dIII.

Southern analysis of injected DNA

After the injected individuals reached the third instar larval stage, DNA was isolated and digested with *Eco*RI. After Southern blotting, filters were probed with a 2.2 kb *Hpa*I-*Xba*I fragment. We looked at the proportion of surviving catenane and parent material (pWX3501) after injecting a 2:1 mixture of pWX3501 and supercoiled catenane (Figure 4a, lane 1). Supercoiled catenane components and precursor plasmid survived equally well, as indicated by the fact that the proportion of the two forms in third instar larvae (Figure 4b, Lane 2) approximated their proportion in the injected material.

Separation of the two rings prevents rescue of *Adh*^{S} activity

Catenaned material was digested with *Eco*RI which linearized both rings. The linearized *Adh*^{S} and *Adh*^{F} rings were separated by electrophoresis on agarose gels, religated in separate reactions and injected as a mixture of *Adh*^{S} and *Adh*^{F} containing rings. One hundred and twelve third instar larvae were individually assayed by electrophoresis. Eighty-three had ADH^F activity but none showed ADH^S activity. Thus a plasmid carrying the NS1-deleted *Adh*^{S} gene could not support enzyme activity when it was not interlocked with a plasmid carrying the *Adh*^{F} gene at the time of injection. These results eliminated the possibility that the BOP′ sequences in the ring carrying the NS1-deleted gene were responsible for the restoration of activity.

DISCUSSION

Our aim was to construct a plasmid that was capable of recombining *in vitro* to produce two interlocking circles, each circle containing a different *Adh* gene. One gene would be defective, lacking a critical enhancer region. Previous studies had shown that the activity of the defective gene could not be restored by the presence of a second plasmid containing an intact *Adh* gene injected at the same time. But activity could be rescued by the presence of an intact gene and a defective gene on the same plasmid. We wanted to determine what would happen if the two genes were injected on separate, but interlocking, DNA molecules.

In this report, we show that activity can be restored to a defective gene by an intact one when the two genes are held in close proximity but not on the same DNA molecule. The restoration of activity in this case can be as effective as the rescue that occurs when the two genes are on the same plasmid. Moreover, because the proportion of heterodimer is near to that predicted by random association of monomers (Table 1, column 3), we can deduce that the two genes appear to be active in the same cells at about the same time.

Although activity was observed when either nicked or supercoiled catenanes were injected, the supercoiled preparation was much more effective in restoring ADH^S activity (S/F ratios approaching 1). In addition, the nicked preparations yielded S/F ratios that varied much more than the supercoiled ones (Table 1). The differences between these results could have been due to differences in linking number among the different preparations, or to an increased susceptibility of the nicked plasmids to nucleases (ADH^S activity would be sensitive to destruction of either ring; ADH^F activity would only be reduced if the ring containing the ADH^F gene were disrupted).

It is unlikely that the observed results are due to a reversal of catenane formation after injection into the fly (i.e., reformation of a single circle containing both genes by recombination). As shown in Figure 4, Southern analysis of larvae injected as embryos with a 2:1 mixture of pWX3501 and catenane revealed about the same ratio of the two rings to unrecombined material after 6 days of residence in the fly. Thus the two rings and pWX3501 seem equally stable after injection. On the other hand, we have not demonstrated that the two rings of the catenane remain interlocked after injection. However, when the two rings of the catenane were injected as a mixture of separated rings, there was no restoration of activity of the NS1-deleted gene. Activation of the NS1-deleted gene required that the two rings be interlocked at the time of injection. However, there remains the real possibility that decatenation may occur shortly after injection as it does in *Xenopus* eggs (26), but that the two rings remain associated by interactions between proteins bound to the enhancer and the promoter regions of each gene.

Our observations are most consistent with loop formation (27) or 'looping' (5). In the looping model the protein or proteins bound to the enhancer are able to associate with a set of proteins bound near the site of the start of transcription to form a transcription complex. Presumably, this association cannot occur in *trans* because the two sites are not close enough to encounter each other. The expectation is that this association can occur in *trans* in a catenane because the two sites are in proximity. Similar conclusions have been reached by others. Schaffner and coworkers (28) bridged the SV40 enhancer to the β-globin promoter via an avidin or streptavidin protein. Dunaway and Dröge (26) used a system that was similar to that described here except that the enhancer was from a polymerase I gene and they worked with *Xenopus laevis* eggs. Finally, Wedel *et al.* (29) used resolvase from the transposon Tn3 to produce two interlocking rings in order to study the interaction of proteins bound to the NtrC upstream binding site and the *gln*A promoter.

Our experimental design differs from that of the above studies in one important respect: The enhancer element that we tested in *trans* was already associated in *cis* with an intact gene (The other cited studies utilized an isolated enhancer element on one plasmid and the gene that it activated on another.) The rationale behind our design was that we could easily quantitate the activity of the defective gene relative to that of the intact. The results that we obtained using this configuration contrast with those from a recent study (30) of the molecular basis of transvection, a pairing-dependent regulatory phenomenon observed in *Drosophila* (31−33). Geyer *et al.* (30) demonstrated that an enhancer of a gene carrying a nonfunctional promoter was able to *trans*-activate a paired homologous gene that carried an inactive enhancer element (thereby providing a model for the phenomenon of transvection). The enhancer's ability to *trans*-activate was eliminated when its intact cognate promoter was present in *cis*. In their system, the enhancer interacted with a functional promoter in *cis* at the expense of *trans* activation. In ours, the presence of an intact promoter did not eliminate the ability of an enhancer to *trans*-activate a defective gene. The fact that the genes that Geyer *et al.* worked with were located on homologous chromosomes while ours were on interlocked plasmids may be responsible for this discrepancy.

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