Transcription at the ecdysone-inducible locus 2B5 in *Drosophila*

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ABSTRACT

The Broad-Complex (BR-C) of *D. melanogaster*, mapping at the 2B5 early ecdysone puff, mediates ecdysone-induced processes. We present here the transcriptional analysis of the locus in both wild type and representative mutants. Two well defined regions are transcribed, only one of which has a pattern consistent with the proposed 2B5 located BR-C function. The 2B5 region is actively transcribed in early third instar larvae before ecdysone levels increase. Ecdysone switches this early pattern to a complex late type which requires the presence of functional BR-C product. Therefore, BR-C self-regulates its own ecdysone-induced transcription. The effect of 2B5 mutations on transcription at several intermolt, early and late puffs is also described. Null mutations at the 2B5 locus prevents ecdysone inducible transcription. This evidence supports the fact that 2B5 codes for an ecdysone-dependent transcriptional regulator.

INTRODUCTION

At the beginning of the third larval instar of *D. melanogaster*, some prominent puffs (intermolt puffs, eg 68C) can be observed, defining the so-called puff stage 1 (PS1). Some hours before pupariation, a rise in ecdysone titre induces dramatic changes in puffing activity. A few minutes after this increase, the early puffs appear and the intermolt puffs regress. Early puffs are active for about 4 hours and, after reaching their maximum size, regress and are substituted by the late puffs [1]. The entire cycle of activation/regression involves at least 125 puffs in a very precise and coordinated way. This process, can be reproduced *in vitro* by incubating dissected salivary glands in the presence of 20-OH ecdysone [1].

One of the very first regions to puff upon contact with ecdysone is the 2B5 site at the tip of the X chromosome. Extensive genetic analysis [2, 3, 4] has lead to a wide collection of mutants mapping at this early puff. The 2B puff contains a genetic locus, now called the Broad-Complex (BR-C) [2, 5, 6], that has a complex complementation pattern. BR-C encompasses two independently mutable functions, br^+ and $1(1)2Bc^+$ [2, 6]. Both functions are abolished by npr-1 mutations. BR-C deficiencies [2, 7, 4, 8], allow normal development until third larval instar but no detectable ecdysone induction is present. Administration of the hormone does not induce further development [9].

Physiological [8, 10] and genetic [2, 6, 7] evidence suggest that BR-C plays an important role in the process of metamorphosis. The locus itself is large (over 100kb) with some of the associated RNA bands being ecdysone inducible [11, 12, 13, 14, 15].

We have already described the molecular pattern of 2B5 (BR-C) mutations [16]. Here we report the transcriptional analysis of this locus and its relationship with other loci present in intermolt, early or late puffs. 2B5 has a rather complicated pattern of expression. Analysis of the transcription pattern of the mutants may elucidate the role of the locus during metamorphosis in *D. melanogaster*.

MATERIALS AND METHODS

Flies

Most of the stocks used in this work have been described previously [2,3, 17, 18]. $br^{32/10B}$ is an hybrid-dysgenesis induced br lethal allele obtained from I. Zhimulev. br^{x195} and $npr\text{-}1^{x1002}$ mutants were isolated in our laboratory by X-ray saturation screening (4000 R).

Embryos were collected from population cages [9].

Early third instar larvae (LIIIE) were obtained from a 2 hour lay that was allowed to develop in bottles without overcrowding. Every hour, 90 hours after laying, all larvae on the bottle walls were collected and considered as PS1 larvae [20]. Squashing of the salivary glands was performed upon a representative sample to confirm correct staging.

Lethal group larvae were obtained from the appropriate crosses, sexed and identified according to larval markers such as yellow 'y' [17].

Pupae were staged as previously described [21].

RNA extraction

Whole organism RNA was extracted according to Crowley *et al.* [23]. Poly(A)$^+$ RNA was selected using oligo (dT) cellulose (Boehringer) under conditions suggested by the supplier. RNA was subjected to electrophoresis in 2.2 M Formaldehide, 1%

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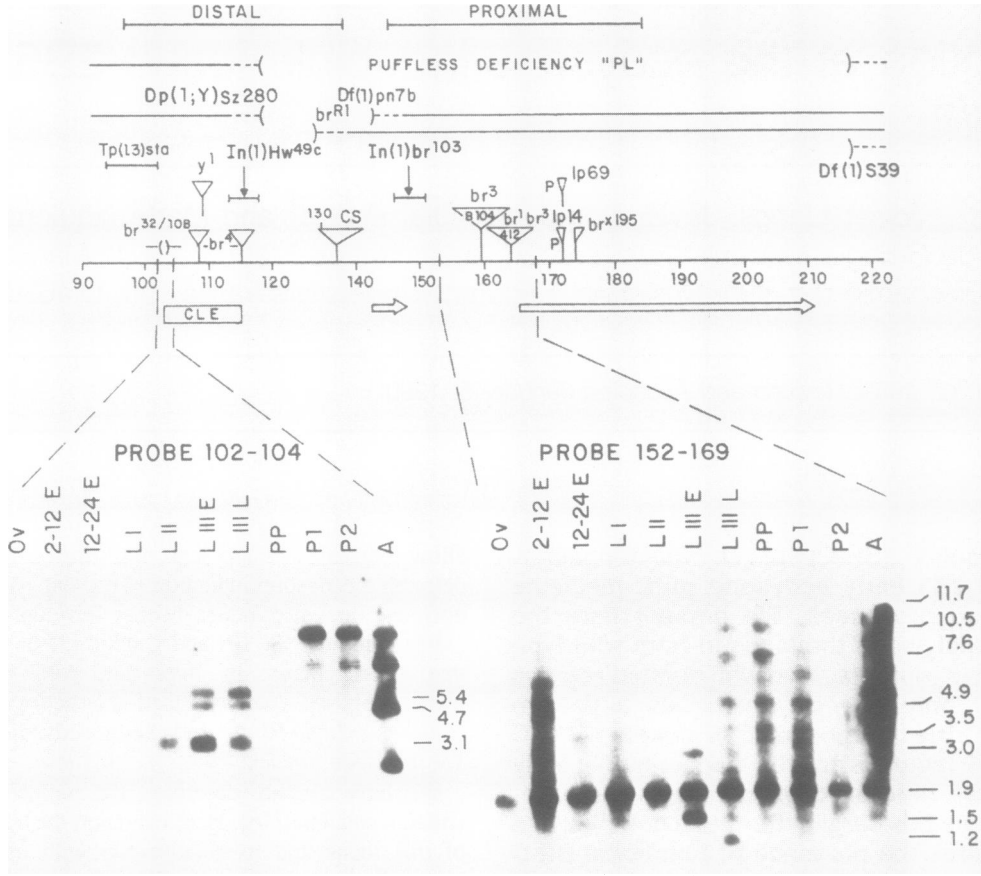

Figure 1 Scheme of the mutation map of the 2B5 ecdysone locus [16] and transcription from distal and proximal regions. Breakpoints are shown as vertical arrows, deficiencies as parentheses, with solid lines indicating DNA presence and dashed lines indicating uncertainty. A repetitive element present in Canton S and other stocks (coordinates 129−137) is indicated by a triangle labelled CS. The open arrows represent the approximate location and orientation of transcription. CLE represents a cis-acting long distance element [16]. 5 μg of poly $(A)^+$ RNA from canton-S of different developmental stages was hybridized to a distal probe (left) or proximal probe (right); (coordinates 102−104 and 152−169 respectively). RNA was isolated from ovaries (OV); 2 to 12 hour embryos (1−12E); 12 to 24 hour old embryos (12−24E) first, second and third instar larvae (LI; LII; LIII); prepupae (PP); pupae (P1), late pigmented pupae (P2) and adult (A). Left and right Northerns are the same filter. The 1.9 kb constitutive band is considered as internal control to calibrate the comparative amounts of RNA.

agarose gels [24], electrotransferred to Nylon membranes (Nytran, Scleicher and Schuell) and hydrolized according to standard procedures [25].

The primer extension procedure was performed essentially as previously described [26]. 6 μg of poly(A)$^+$RNA from early larvae was annealed to 5 pmol of labelled genomic DNA from coordinates 146−147, 147−148 and 148−151 (Figure 1). RNA from late larvae was annealed to 5 pmol of labelled DNA between coordinates 165 and 165.5 in 0.1M Tris-HCl at pH 8.3, O.14 M KCl and 1 mM EDTA by heating to 85°C for 5 min and the mixture incubated at 42°C for 1 hour. The total volume of this reaction was 28μl. At the end of the annealing period the mixture was adjusted to 60 μl containing 0.5 M each of dCTP, dATP, dGTP and TTP, 15 mM Dithiothreitol, 10 mM $MgCl_2$, 25 units of placental ribonuclease inhibitor and 50 units of AMV reverse transcriptase (Seikagaku America, INC.). Reverse transcription was then carried out at 42°C for 1 hour. The reaction was stopped by adding 1.5 μl of 0.5 M EDTA at pH8. The nucleic acids were precipitated with ethanol, harvested by centrifugation and dissolved in 10 μl of 80% formamide containing 50 mM Tris-borate (pH 8.3), 1 mM EDTA, and 0.1% (W/v) each of xylene cyanol and bromophenol blue, and resolved on polyacrylamide-urea sequencing gels [27].

RESULTS

We have examined the transcription pattern of 2B5 (BR-C). All the mutants mapped by us [16] cluster in two separate regions called distal and proximal (with respect to the centromere).

The distal region spans sequences between Df (1:3) sta and Df(1) pn 7b (approximate coordinates 100−145). This part of the complex is required for BR-C$^+$ and therefore defines the left end of the complex. The proximal region spans sequences between Df(1) pn7b and S39 (approximate coordinates 145−215); again alteration results in complete loss of BR-C function [14, 16].

A representative Northern for both distal and proximal regions is shown in Figure 1. The distal region shows very little transcription in embryogenesis, increases in larvae, changes pattern in pupa and keeps the pupal transcription incremented with new small size RNA classes in adults. The pattern seems to be developmental stage specific but not ecdysone triggered. Ecdysone titres increase drastically between early and late third instar larvae (LIII E and L III L in Figure 1 left panel); however no significant differences between early and late transcription occurred during larval stages.

The proximal transcription, on the other hand (Figure 1 right

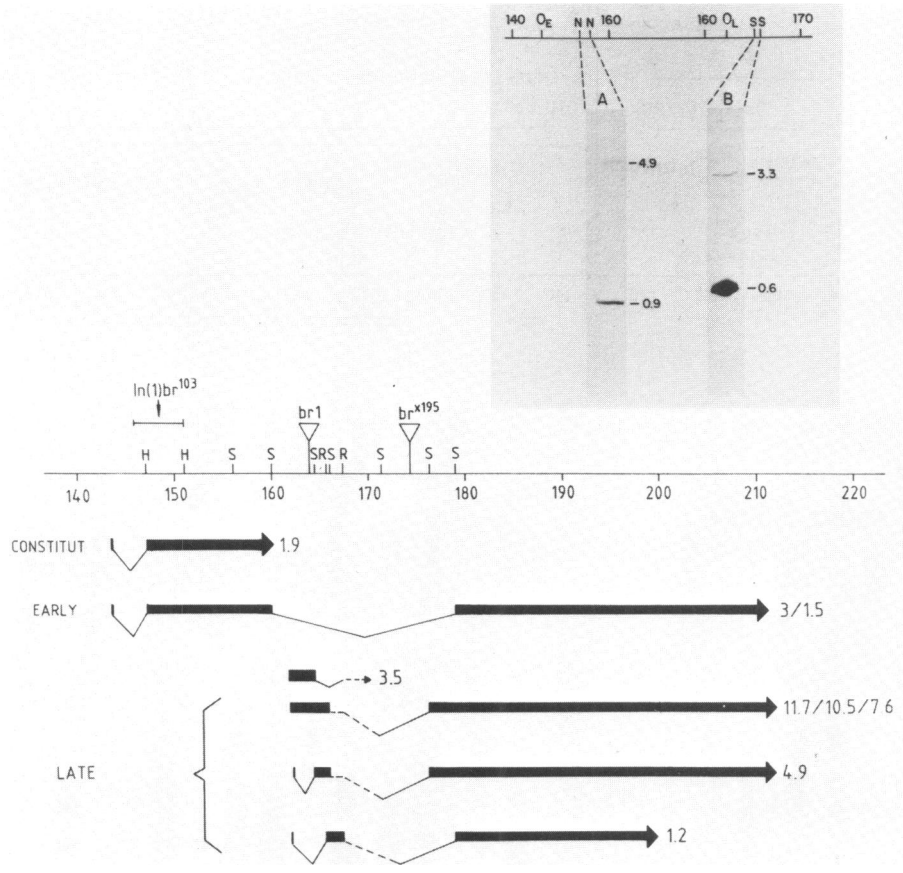

Figure 2 Primary extension example (upper right) and schematic diagam of transcription at the points were determined by primary extension (uppper right); primers of 0.9 and 0.6 were obtained by Bru I (N) and Sal I (S) digestion and were exteded to 4.9 and 3.3 respectively defining the early RNA initiation site (O_E) at coordinate 143 (A) and the late RNA initation site O_L at 165 (B). At the bottom diagrammatic representation of transcription, the direction of which was known (11). Untranscribed regions have been deduced by RNA negative hybridization to small genomic restruction fragments (with an approximate error of 0.1 to 0.5 kb). Dashed lines are regions with uncertainties.

panel) clearly shows a significant difference between early and late larvae with the appearance of three very prominent bands of high molecular weight (10.5, 7.6 and 4.9 kb) and other minor bands (11.7 kb for example). One conclusion is that the proximal region harbours the ecdysone inducible product of BR-C complex.

Northern blots were probed with small specific subclones (0.5 to 5 kb) from almost the whole proximal region (date not shown). Results indicate that there is a complex transcriptional pattern with several alternate exons or termination sites (Figure 2). Early and late larval bands can be detected between coordinates 150 and 210, but only the former are detected between coordinates 140 and 160, indicating the existence of two different initiation sites for early and late transcripts. In order to confirm this prediction primer extension experiments were performed. The early RNA initiation site was located by using three primers mapping between coordinates 146−147, 147−148 and 148−151, respectively. All of them indicated that the early RNA initiation site is located at coordinate 143 (Figure 2 upper right). With regard to the late RNA in initiation site, we used as primer a 0.6 kb genomic fragment mapping at coordinate 165. The late RNA transcription starts at coordinate 162 (Figure 2, upper right).

Transcription in BR-C mutants

Having established the transcription pattern in the wild type, a study of the alterations produced by representative BR-C mutations was undertaken. Considering that loss-of-function (npr-1 mutants), as well as puffless males, died at early LIII, we tried to look at the early to late LIII transition.

Poly(A)$^+$ RNA was isolated from early and late hemizygous third instar larvae (LIII) bearing representative BR-C mutations, and probed for proximal and distal transcriptional alterations.

To assess transcription of the distal region in BR-C mutants, the same probe as in wild type individuals was employed (coordinates 102−104) (Figure 3). The two null alleles npr-1^{x1002} and npr-1^{324}, both apparently due to point mutations (unpublished observations) showed an unexpected result: third instar larvae lack the 4.7 kb band and some new bands not detected in wild type are apparent. These two mutants die in early LIII, and never show the puff activation-inactivation cycle characteristic of late LIII. br$^{32/10B}$, similarly to npr-1^{324}, shows new bands not detected in wild type, less prominent in late larvae. br^1 does not show apparent alteration from wild type. In (1) Hw^{49c} [28, 16], a viable br mutant, shows the absence of 5.4 kb band. In view of its viability, this RNA class should not code for any vital function.

The proximal region (Figure 3), npr-1^{x1002} and npr-1^{324} shows an unchanging early transcriptional pattern without any sign of the late bands. npr-1x^{1002} also shows reduction or even loss of the 3.5 kb band (being very weak in the Canton S sample is difficult to ascertain). In (1) br^{103}, a br lethal dying in early pupa, shows early and late bands similar to wild type. Therefore,

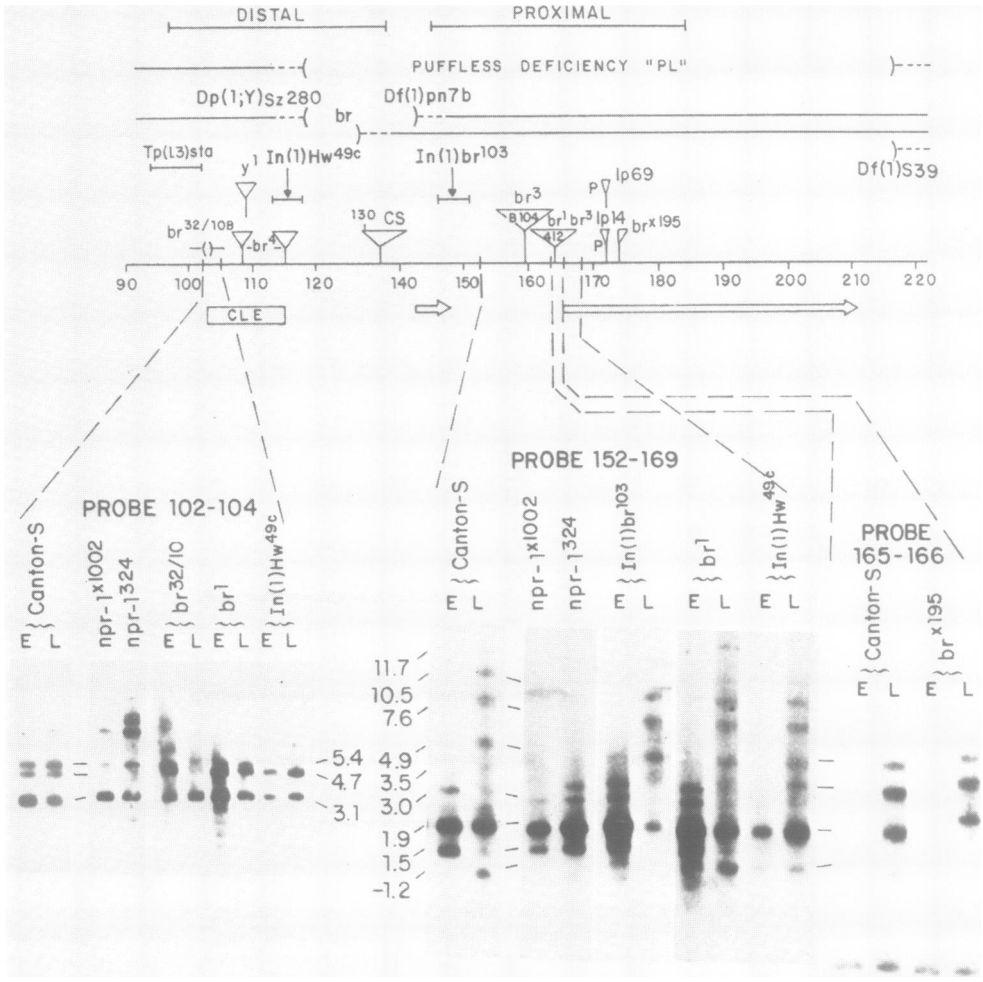

Figure 3 Transcription in mutant third instar larvae. RNA from different mutants was analyzed by Northern blots. Early (E) and Late (L) larvae RNA are shown for wild type samples (Canton S) and some of the mutants. At the bottom of the further right Northern is shown the hybridizations with β-tubulin DNA to measure RNA content of the filters. The other filters show internal constitutive bands as controls for RNA concentration.

this inversion seems not to grossly disrupt any of the transcripts. br^{x195} carries a small insertion in coordinate 174 [16] and dies as prepupa. Its RNA shows a clear increase in the size of all the late transcripts, as can be seen in Figure 3.

Analysis of the transcription pattern in other mutants show no clear differences compared to wild type. Examples being the br alleles br^1 and $In(1)Hw^{49c}$, both viable and fertile, do not show any gross alterations.

Relationship between BR-C and other loci

BR-C has been described as an important locus for ecdysone response in LIII, and there is some evidence for BR-C to be required for expression of other loci [7, 23]. Here attempts have been made to clarify the effect of BR-C in this cascade by use of three null BR-C mutants ($npr\text{-}1^{x1002}$, $npr\text{-}1^{324}$ and a BR-C-deficiency) and probes from intermolt, early and late puffs (Figure 4).

Intermolt puffs

It has been previously shown [23] that npr-1 individuals fail to transcribe Sgs-3, a salivary gland glue gene mapping at the 68C intermolt puff. Figure 4 confirms those findings (though some Sgs-3 signal is yet detected in very long exposures of these Northerns date not shown). Sgs-4, another salivary gland glue gene mapping at the 3C intermolt puff [29, 30], also shows a dramatic reduction in puffless larvae, in agreement with previous work [23].

Early puffs

The effects on early puffs has been studied on three well known puffs: 74EF, 75B and 2B itself. Probe G1 from 74EF detects a fat body specific RNA which is not induced with ecdysone. As can be seen in Figure 4c, BR-C deficiency (PL) does not affect transcription of this RNA. Probe C74, also from 74EF, detects three different bands in whole organism RNA, only one of which (1 in Figure 4d) is ecdysone-inducible. Effectively, by comparing the amount of RNA loaded in early and late Canton S lanes (Figure 4g), it is clear that this band shows a higher transcription or stability in the late lane. This band appears at early, uninduced levels in BR-C mutants. Probe 8B6 from 75B is complementary to several RNA bands, one of which, sized 6.5 kb (arrow in Figure 4e), is only present in late LIII. This band cannot be detected in puffless, $npr\text{-}1^{x1002}$ or $npr\text{-}1^{324}$. As noted above, BR-C null mutations affect late BR-C transcription itself, which could be considered another case of effect on early puffs.

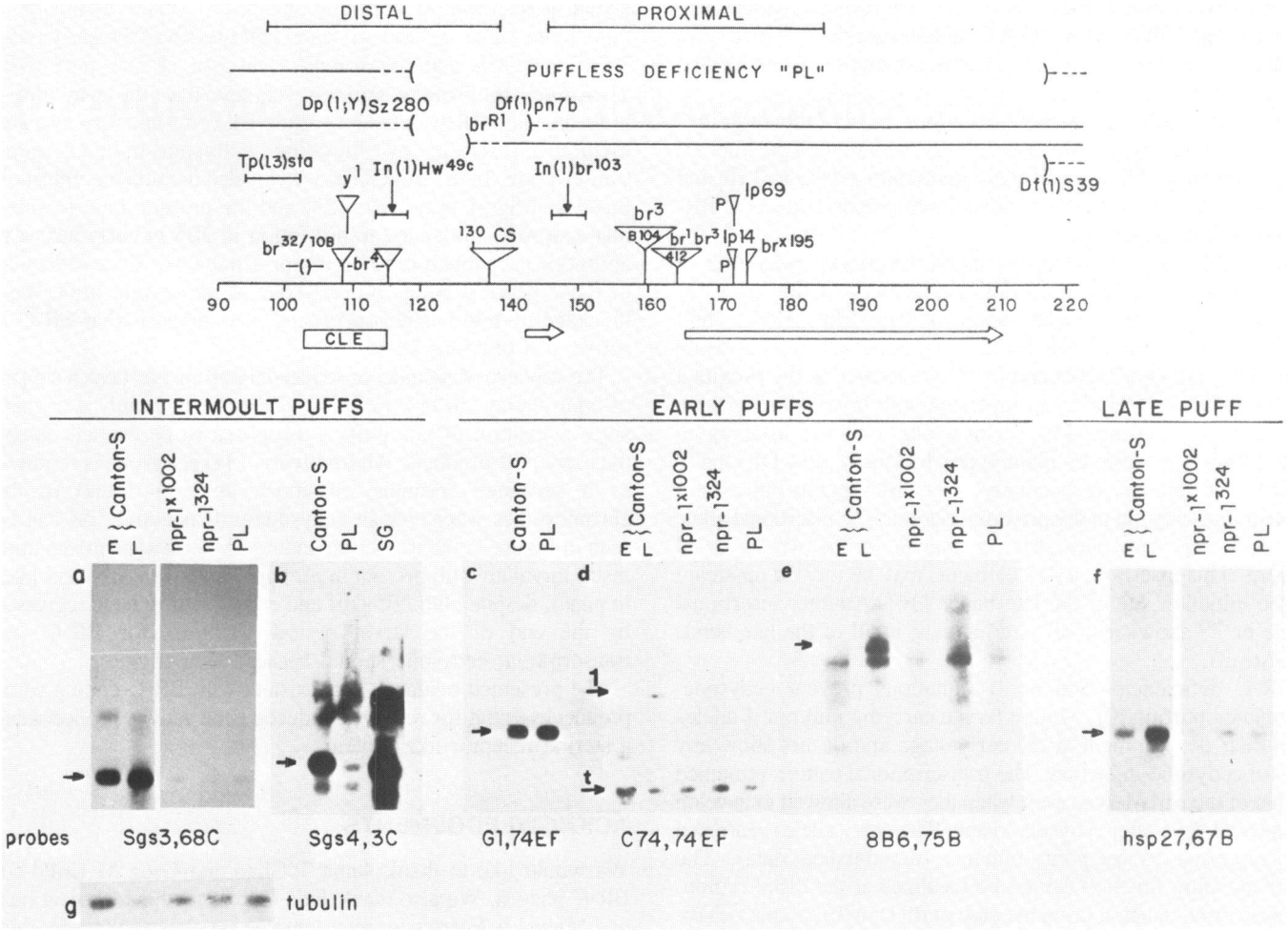

Figure 4 Effect of 2B5 mutations on transcription at other loci. About 5 μg of poly(A)$^+$ RNA was loaded on each lane. The puffless deficiency (PL) is heterozygous Df(1) S39/Dp(1;Y)Sz280 lacking all proximal part of the BR-C. Wild type (Canton S) early (E) and late (L) RNAs are shown to the left of mutants. a) Probe: Sgs-3 from intermolt puff 68C. b) Probe: Sgs-4 from intermolt puff 3C. SG, RNA from isolated salivary glands. c) Probe: G1 from early puff 74EF. d) Probe: C74 from early puff 74EF. Arrow marked 't' indicates the hybridization band to a β-tubulin plasmid. Arrow 1 marks the inducible RNA class, arrows in a) and c) indicate other bands not affected by the hormone. e) Probe: 8B/6 from early puff 75B. Arrow points to the inducible RNA class. f) Probe: hsp-27 from late puff 67B. g) Same filter as in (a, e, f) probed with β-tubulin DNA (a, d, e, f, g) are the same filter.

Late puffs

One well characterized late puff is 67B, in which the hsp27 heat-shock gene is located [31]. As with other minor heat-shock genes, hsp-27 is ecdysone inducible. As can be seen in Figure 4, its transcription is induced in wild type, but is very much reduced in BR-C mutant late LIII (very long exposures of the Northern show a very reduced expression, data not shown).

DISCUSSION

BR-C has been described as one of the genes controlling the larval/prepupal/pupal transition [7, 4]. Evidence for the implication of BR-C product in the control of ecdysone-induced gene activity is based upon disruption of puffing pattern [7, 32] and abolition of transcription in some intermolt puffs (like 68C in BR-C deficient mutants) [23]. According to the RNA pattern, two well defined regions can be distinguished in the mutationally defined BR-C locus.

The developmental profile in the distal region, which includes transcripts in all embryonic, larval, pupal and adult stages, is not consistent with the known lethal phases of BR-C mutations. Expression in that region seems not to be affected by the hormone: there is not any appreciable difference between early and late larval RNA pattern. Indeed, those transcripts are not expected to be involved in BR-C function, since the distal region is not functional when contributed in trans [16]. It has been proposed that it bears a long distance, cis-acting element, that enhances expression of the proximal transcriptional unit. In this context, the observed alteration of the distal RNA bands in some mutants, including two punctual npr-1 alleles, is rather an unexpected result. However, they can be seen not as a cause, but as an effect of the mutations. Distal transcription, therefore, could be regulated by BR-C products.

In the proximal region, some bands also show unexpected profiles. First, a 1.9 kb band is expressed constitutively. This band is only detected about coordinates 140−160. Second, a minor 3.5 kb band is expressed from ovaries and embryo to early third instar larvae. The presence of maternally inherited transcripts may be irrelevant since experiments involving germ-line clones have shown no maternal effects for npr-1 lethal phase [33]. It is of course conceivable that BR-C is performing some non-essential function in former stages similar to its regulation

of Sgs-3 expression in the early third larval instar. It should also be mentioned that many BR-C mutants develop slower than controls, especially in overcrowded population (unpublished observations).

Despite the above mentioned exceptions, most of the transcripts in the proximal region exhibit profiles consistent with the inferred BR-C function. They reach their maximum levels in LIII and prepupal stages, when all described developmental effects of BR-C mutations do occur.

Difficulties arise in trying to match the genetic behaviour of BR-C to the complex transcriptional activity of the locus. br alterations span over 70 kb. Some of them, In(1)Hw^{49c}, br^{4}, br$^{32/10B}$, for example, are located at the distal region while In(1)br^{103}, Df(1)pn7b, br^{1} and br^{x195} are located at the proximal one [14, 16]. In(1)Hw^{49c}, an inversion with breakpoints in suite and at BR-C coordinate 115, seems to alter only the distal region and it shows a weak br phenotype; In(1)br^{103} and Df(1)pn7b splices and deletes, respectively, the distal region but do not abolish transcription in the proximal region. The insertional allele br^{1} does not show any striking change in the whole larval pattern. This insertion, a 412 element, may be located upstream to the initiation site of the late bands [16]. Another insertional allele br^{x195} shows a small size increase in all of the late bands (Figure 3).

BR-C deficiencies and npr-1 mutations prevent ecdysone-stimulated puffing [7]. Mutant larvae carrying null npr-1 alleles stop their development at the early stage and do not show any sign of ecdysone-induction. The transcriptional pattern remained as that of the early larvae even when they were allowed to develop for several days after ecdysone rising. The npr-1 alleles examined are supposed to be point mutants (unpublished data). The corresponding function cannot be localized at the distal region, because distal deletion (as in the case of df(1)pn7b), do not behave as a npr-1 alleles. On the other hand, a deficiency removing sequences to the right of coordinate 175 completely abolished all of the BR-C function [14]. This suggests that the proximal region harbours the BR-C function.

Proximal region presents a rather complex transcriptional pattern: early and late larval bands can be detected between coordinates 160 and 210, but only the former are detected between 150 and 160, indicated the existence of two different initiation sites for early and late transcripts confirmed by primer extension experiments. However, small exons may have gone undetected.

The prevention of ecdysone-stimulated puffing in npr-1 mutations includes that of the early puffs, whose induction is known to be independent of de novo protein sythesis [1]. Therefore, BR-C products involved in early puff activity must be present in the cell before ecdysone rising. What is rather puzzling is that the BR-C locus is itself an early ecdysone-induced puff. This paradox is only apparent since, as shown in Figure 1, BR-C is actively transcribing some RNA classes in early larvae, well before ecdysone rising and independently of the 2B puff stage. As noted above, ecdysone switches the BR-C pattern to a late type, probably in inducing transcription from a different promoter. This induction is mediated by the BR-C products itself, since the two punctual npr-1 alleles examined: npr-1^{324} and npr-1^{x1002} lack only the late larval RNA bands. The developmental arrest in npr-1^{x1002} is shown in Figure 3 where no signs of the late bands are detected. BR-C late bands are not the only ones to be affected by npr-1 mutations. All the examined transcripts mapping at ecdysone puffs and showing increased levels in response to hormone also show sensitivity to npr-1 mutations. These include one transcript in each of the early puffs 74EF and 75B and the hsp-17 transcript at late puff 67B. Therefore, the results strongly suggest that the way npr-1 mutations and BR-C deficiencies prevent ecdysone response and metamorphosis is by blocking ecdysone-induced gene transcription. In the same line, we can also include the intermolt Sgs-3 and Sgs-4 transcripts [23] and the present data, to show that ecdysone stimulated transcription in 2B5 in early larvae is absent or very much reduced in npr-1 mutants. Since induction of those genes depends on ecdysone at the second molt, their blockage by npr-1 is not surprising, and suggests that BR-C is active at a previous stage.

The way ecdysone induces sequential puffing has been a subject of controversy. In one view [1], ecdysone is mainly a trigger, since activation of late puffs is proposed to be carried out by the early puff products. Alternatively, [34] ecdysone is required as a sustained stimulus, inasmuch as it is found in the chromosomes whenever an ecdysone puff is active. As can be seen in Figure 1, the transcripts induced by ecdysone in late third instar larval are still present in prepupa (and even two days later in pupa), despite both 2B5 puff and ecdysone titre have regressed by the end of the larval period. At least for BR-C late transcription, ecdysone should be seen as a trigger.

The presented evidence is consistent with BR-C coding some product essential for ecdysone-induced gene activity, conceivable a steriod receptor-like protein.

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