The association of transcribed genes with the nuclear matrix of *Drosophila* cells during heat shock

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Received 21 November 1984; Revised and Accepted 11 March 1985

ABSTRACT

Using the transcriptional modulation afforded by heat shock, we found that the association of active genes with the nuclear matrix was not dependent on their level of transcription. Heat shock genes were matrix associated both before heat shock (when transcription was relatively low), and during heat shock (when transcription was greatly increased). Conversely, the cytoplasmic actin gene was matrix associated during normal growth conditions (when transcription was high) and during heat shock (when transcription was greatly decreased). Removal of greater than 99.7% of nascent RNA during preparation of the matrices did not affect these findings. Detailed examination of the cytoplasmic actin gene revealed that its matrix association was apparently mediated by multiple interactions near the 5' end of the gene.

INTRODUCTION

One of the most active areas of molecular biological research is dissection of the factors controlling transcription in eukaryotes. Through these studies, it has become clear that the conformation of DNA sequences in the intact nucleus plays an important role in gene expression. Numerous differences between the nuclear conformation of transcriptionally active and inactive DNA sequences have been identified. These differences include location in euchromatin versus heterochromatin, increased sensitivity of active genes to digestion by nucleases, an altered nucleosomal structure of active genes, and their association with specific proteins (1,2).

Another recently described difference between inactive and active genes is the association of active genes with the nuclear matrix. This structure has been defined in two ways. Morphologically, the structures remaining after extraction of nuclei with salt to remove most histones and other proteins appear to represent a proteinaceous network of fibers as observed by electron microscopy (3-5), although the composition and morphology of these structures has engendered considerable controversy (6,7). Operationally, it has been found with biophysical and biochemical

techniques that supercoiled loops of DNA appear to be anchored to a salt insoluble nuclear substructure (8-11). This substructure apparently includes the proteinaceous network described by electron microscopic experiments, but the exact relationship between the nuclear elements responsible for anchoring DNA loops and those observed by electron microscopy remain conjectural. In this manuscript, the nuclear matrix refers to an operational entity encompassing those salt insoluble elements which appear to anchor DNA loops. Previous studies have shown that a variety of genes in mammalian and avian species are associated with such nuclear matrices in cells in which they are transcribed (12-17).

An increased understanding of the factors involved in determining the nuclear conformation of active genes can be gained by manipulating the transcriptional state of the gene and monitoring the effects of this manipulation on the gene's nuclear conformation. Heat shock has been a useful experimental tool in this regard. When *Drosophila* flies, embryos, or tissue culture cells are removed from their normal environmental temperature and placed instead at 37°C, a distinct set of DNA sequences (those coding for the heat shock proteins [hsp]) rapidly become transcribed at a greatly increased rate, while transcription of other genes is reduced to very low levels (18,19). Immunofluorescence studies using antibodies to RNA polymerase II to stain polytene chromosomes have shown that most of the RNA polymerase molecules previously bound to transcribing regions are released by heat shock, and much of this polymerase subsequently binds to the heat shock loci (20,21). Because of the rapid, qualitative alterations in gene expression afforded by heat shock, this has been used to study many aspects of gene expression, particularly the nuclear conformation of genes as monitored by sensitivity to nucleases. Such studies have indicated that the general nuclease sensitivity of active genes markedly changes during heat shock, while the DNAase I hypersensitivity of certain DNA sequences surrounding these active genes is invariant during low or high states of transcription (22-24).

In this study, we have used heat shock to determine whether the drastic changes in levels of transcription observed as a consequence of this treatment alter the association of active genes with the nuclear matrix. We found that heat shock did not alter the association of active genes with the nuclear matrix. Hence the matrix association of active genes, like DNAase I hypersensitivity, was not dependent on high rates of transcription. Additional studies described below showed that (1) the

matrix association of active genes was not mediated through nascent RNA and (11) the matrix association of the cytoplasmic actin gene was mediated through multiple interactions near its 5' end.

MATERIALS AND METHODS

Cells

The *Drosophila* Kc cell line (25) was grown in D-22 media (26) at 23°C with 10% fetal calf serum plus antibiotics. Cells for nuclear matrix preparations were grown in the presence of ³H-labeled deoxythymidine (0.1-0.5 uCi/ml) for several days in either T-flasks or spinner flasks. They were harvested in late log phase. For heat shock, cells were gently shaken at 36.5°C for 30 minutes prior to harvesting.

Preparation of Nuclear Matrix DNA

Cells were washed in Hanks' buffered saline and then resuspended at 1x10⁷/ml in IS Buffer: (100 mM NaCl, 50 mM KCl, 5 mM MgCl₂, 20 mM Tris pH 7.2, 0.5 mM phenylmethylsulfonyl fluoride [PMSF], 0.2% methylmethanethiolsulfonate [MMTS] [16]). 1/10 volume of 10% NP-40 in IS buffer was then added while swirling the cells. Nuclei were pelleted by centrifuging at 400 g for 5 min and brought up in the same volume of IS buffer plus 1% NP-40. After ten strokes in a Dounce homogenizer with a tight fitting pestle, the nuclei were again pelleted. The nuclei were resuspended in IS buffer at 2-3 x 10⁶/ml and an equal volume of 4M NaCl, 20 mM Tris, 5 mM MgCl₂, 0.5 mM PMSF, 0.2% MMTS was then added to the nuclei. The resultant "matrix-halo structures" (see results) were centrifuged through 4% glycerol in HS Buffer (2.0 M NaCl, 5 mM MgCl₂, 20 mM Tris pH 7.2) at 1500 g for 15 min onto a cushion of 60% glycerol in HS Buffer. The structures were removed with a Pasteur pipet and diluted 20-40 fold with 100 mM Tris pH 7.2, 5 mM MgCl₂, 2 mM B-mercaptoethanol. The matrices were then digested with 20-40 units/ml of EcoRI and/or other restriction endonucleases (all from BRL, Bethesda, Maryland) at 37°C for varying times at a density of 0.5-1 x 10⁶ structures/ml. After digestion, matrices were pelleted at 10,000 xg for 20 min. DNA extraction, agarose gel electrophoresis, Southern blotting and hybridization, were carried out as previously described (15). Results were quantitated with a Clifford Densitomp model 445 densitometer and a Hewlett-Packard 9874A digitizer.

In vitro transcription analysis

In vitro transcription of isolated nuclei (27-29) was used to directly measure the transcription rates of the various genes used in this study.

Briefly, nuclei were suspended in transcription buffer (0.6 mM ATP, 0.3 mM GTP, 0.3 mM CTP, 3.0 mM dithiothreitol, 1.2 mM MgCl₂ and 80 mM KCl), 100 uCi of 32P-UTP [Amersham, 3000 Ci/mmol] was added and transcription was allowed to proceed for 15 min at 23°C. The reaction was stopped by incubation with RNAase free DNAase I (Worthington DPRF grade) followed by a one hour incubation with 100 ug/ml proteinase K. After extraction and ethanol precipitation, 32P-labeled nascent RNA (50 x 10⁶ dpm) was hybridized to nitrocellulose filters containing plasmid DNA. After washing, filters were autoradiographed and then bands were excised and counted. The data for percentage of nuclear transcripts corresponding to each gene has been corrected for efficiency of hybridization (25% as determined with 3H labeled RNA standards) and percentage of total transcription made by RNA polymerase II (approximately 40% as judged by alpha amanitin inhibition).

Cloned DNA fragments

The plasmid clones used for hybridization analyses were as follows. For the hsp 70 gene, the cDNA clone adm87A+C.1 was used (30). For the hsp 23 gene, the cDNA clone adm67B.1 was employed (30). For the chorion gene, the cDNA clone pDmc5F9 was used (31). For the cytoplasmic actin gene (located at chromosomal position 5C on the X-chromosome), DmA2, an 8.7 kb EcoRI genomic fragment cloned in pBR322 (32) was used for all experiments except those presented in Fig. 4C. For the experiments presented in Fig. 4C, we used a 3.5 kb EcoRI/SalI fragment subcloned from DmA2 into pBR322. The map in Fig. 4D shows the location of the EcoRI and SalI sites.

RESULTS

When nuclei are extracted with 2M NaCl, the histones and many other nuclear proteins are removed. All of the DNA remains with the residual nucleus, and this DNA can be observed with biophysical or microscopic means to be arranged as a series of supercoiled loops (8,9) anchored to a residual nuclear scaffold or matrix (10,11). In the fluorescence microscope, these loops appear as a fluorescent halo surrounding the matrix ("matrix-halo structures") (10). The supercoiled DNA loops that are anchored to the nuclear matrices can be cleaved with a restriction endonuclease. After centrifugation, detached DNA sequences remain in the supernatant ("supernatant DNA"), while those DNA sequences remaining anchored to the matrix ("matrix DNA") can be recovered from the pellet.

Figure 1A shows a time course of digestion of matrix-halo structures

with EcoRI. Note that in this experiment enzyme was added at time 0 and then the centrifugation to separate the matrix from detached DNA was performed. During the time required for centrifugation, 13% of the DNA became detached. If no enzyme was added, 96-99% of the DNA remained attached to the matrices after centrifugation. The digestion reached a plateau when 24-26% of the DNA remained attached to the matrix. Fluorescence microscopy during this digestion showed that the ethidium bromide stained halo of DNA (originally 6 microns in diameter) gradually decreased in size and intensity until only a relatively faint halo (less than 1 micron in diameter) remained. Note that the 24-26% of nuclear DNA remaining with *Drosophila* matrices represents a higher percentage than that remaining after similar digestion of matrix-halo structures from avian or mammalian nuclei (14,16). This is consistent with the facts that the supercoiled loop lengths in *Drosophila* are smaller than in avian or mammalian cells (33,34) and that *Drosophila* DNA contains a high percentage of satellite sequences which are not cleaved by EcoRI (35). The digestion represented in Fig. 1A was a limit digest, as inferred from two results. First, excess EcoRI added at the plateau point cleaved no additional DNA from the matrices (Fig. 1A). Second, the DNA from the matrix was purified as described in Materials and Methods and a portion of the DNA was fractionated by agarose gel electrophoresis (Fig. 1B, Lane M) next to an equal amount of the same DNA cleaved with EcoRI after DNA purification (Fig. 1B, Lane Mr). After transfer to nitrocellulose, these samples were hybridized with a ³²P-labeled, actin gene probe. As is evident in Fig. 1B, the density of the hybridization bands in the comparable lanes were equal, and there were no bands detected other than the limit digest fragment of 8.7 kb. This was in contrast to lane Mp, which contained an equal amount of DNA from a matrix preparation that was purposely digested partially, leaving 70% of the total nuclear DNA still attached to the matrix. Partial products of digestion were visible in this lane, and the limit digest 8.7 kb species was significantly reduced in intensity compared to the completely digested samples M and Mr. Also evident in Fig. 1B is the fact that the relative concentration of actin gene sequences present in the DNA associated with the matrix (M and Mr) was vastly different from the DNA detached from the matrices (S and Sr).

Using this digestion strategy, we examined the position with respect to the nuclear matrix of a number of genes whose transcription could be modulated by heat shock. We chose for this analysis the genes coding for

Fig. 1A: Time course of digestion of matrix-halo structures with EcoRI. ³H-thymidine labeled matrix-halo structures were incubated at 37° with 5 units/ml of EcoRI. At the indicated times, aliquots were removed and the DNA remaining attached to the matrices was separated from DNA cleaved from the matrices via centrifugation. After 1 hr, a further 20 units/ml of EcoRI were added to the residual structures. Results were expressed as the percentage of total nuclear DNA remaining attached to the matrices after digestion and centrifugation.

Fig. 1B: Assessment of the completeness of digestion of matrix-halo structures with EcoRI. Matrix and supernatant DNA were prepared as described in Methods. Equal quantities (0.5 ug), were fractionated in an agarose gel, transferred to nitrocellulose, and hybridized with a ³²P-labeled cytoplasmic actin probe. Lane M: matrix DNA from 1 hr digestion with 5 units/ml of enzyme. Lane M_r: same sample as lane M but the purified DNA was redigested with EcoRI before loading on the gel. Lane S: supernatant DNA from the same experiment as lane M. Lane S_r: same sample as lane S but the purified DNA was redigested with EcoRI before loading on the gel. Lane M_p: DNA from matrix-halo structures was purposefully digested incompletely by incubation with 5 units/ml of EcoRI for only 3 min.

Fig. 2: Transcription of various genes before and during heat shock.

³²P-labeled nascent RNA was obtained from nuclei as described in Experimental Procedures. 0.5 ug of plasmid DNA containing sequences for the genes listed below were cleaved with EcoRI (to form linear instead of supercoiled molecules) and after electrophoresis through agarose, transferred to nitrocellulose. These filters were then hybridized with the nascent RNA from normal (A) or heat shocked (B) cells. Exposures were for 24 hours (A) or 1 hour (B). The resultant autoradiographs are shown in the figure. Lane 1: chorion; lane 2: actin; lane 3: hsp23; lane 4: hsp70.

hsp 70, hsp 23, cytoplasmic actin, and a chorion protein. Transcription of the hsp 70 and hsp 23 genes has been shown to be remarkably increased by heat shock, whereas the opposite is true for actin genes (19). The data in Fig. 2 confirm these results in Kc cells under our experimental conditions. For this analysis we directly measured the transcription rate of these genes in isolated Kc cell nuclei. The transcription of the hsp 70 and hsp 23 genes was increased by more than 2 orders of magnitude during heat shock. Conversely, actin gene transcription was reduced 8-fold by heat shock. Chorion gene transcription was not detected at either temperature. Quantitation of the results in Fig. 2 showed that transcription of the actin, hsp 23, and hsp 70 genes respectively represented 0.05%, 0.0009% and less than 0.0005% of the nuclear transcripts during normal growth conditions, and represented 0.006%, 0.45% and 1.5% of the nuclear transcripts during heat shock. In other experiments using Western blotting techniques we have determined that there are 10,000 +/- 2,000 RNA polymerase II molecules per diploid Kc cell nucleus (D. Small *et al.*, manuscript in preparation). Hence, the data indicated that during normal growth conditions, less than 1 in 20 cells was actively transcribing each hsp 23 or hsp 70 gene (note that there are 5 hsp 70 genes per haploid genome), while each actin gene was being transcribed by 1 to 2 RNA polymerase molecules. For this calculation we have assumed that all 10,000 RNA polymerase molecules in the nucleus are engaged in transcription and

Fig. 3: The content of specific genes in the DNA from the matrices of normal and heat shocked cells. Matrix-halo structures from *Drosophila* Kc cells were cleaved with EcoRI and the DNA from pelleted matrices was purified and redigested with EcoRI as described in Methods. In some cases, matrix halo structures from control cells were extensively digested with RNase A endonuclease digestion (lanes C_R). Various quantities of EcoRI cleaved total nuclear DNA (lanes marked total), and matrix DNA obtained from control (C) and heat shocked (HS) cells were fractionated by electrophoresis through 0.8% agarose gels and transferred to nitrocellulose filters. Lanes marked 0.5X, 1X, 2X, and 3X contained 0.5, 1, 2, and 3 times the amount of DNA loaded in the lanes containing matrix DNA. Each filter was then hybridized with a ³²P-labeled probe for the following genes:

- (A) hsp70
- (B) hsp23
- (C) actin
- (D) chorion

Fig. 3E: Contents of hsp 70 genes in matrix and supernatant DNA fractions. DNA was purified from matrices (lanes M), supernatant (lane S) and whole nuclei (lanes T) as described in the text. Equal quantities of DNA were loaded in each lane, and after electrophoresis through an agarose gel, the DNA was transferred to nitrocellulose filters and hybridized to the ³²P-labeled hsp70 probe. Lanes Mc, Tc, and Sc were from normally growing cells and lanes Mh, Th, and Sh were from heat shocked cells. The autoradiograph was purposely overexposed to show the small amount of hsp70 genes left in the supernatants.

Fig. 3F: Simultaneous depletion of the chorion gene and enrichment of the cytoplasmic actin gene in nuclear matrix DNA. ³²P-labeled probes for both actin and chorion genes were hybridized simultaneously to a filter containing 1 ug of total nuclear DNA or and matrix DNA.

that the rate of transcription of the individual RNA polymerase II molecules is invariant (i.e., does not depend on the gene). As an example, the number given for the hsp 23 gene was obtained from the following calculation: 0.0009% (percent of total transcription arising from the hsp 23 gene) x 10⁴ (number of RNA polymerase II molecules per nucleus)/2 (number of copies of hsp 23 gene per nucleus) = 0.045, implying 1 of 22 hsp 23 genes was being actively transcribed at the time of measurement. During heat shock, less than 1 in 3 cells was actively transcribing each actin gene, while each hsp 70 and hsp 23 gene was each being transcribed by over 15 RNA polymerase molecules.

To examine the position of these genes with respect to the matrix, we prepared nitrocellulose filters containing matrix associated DNA from control and heat shocked cells and various amounts of total nuclear DNA. These filters were then hybridized with ³²P-labeled clones containing gene sequences coding for hsp 70 (Fig. 3A), hsp 23 (Fig. 3B), cytoplasmic actin (Fig. 3C) and chorion proteins (3D). Fig. 3A shows that the matrix associated DNA from control cells (lane C) was enriched in hsp 70 genes in both 27 and 17 kb fragments. In six independent experiments, this enrichment varied from 3.1 to 3.8 fold. Considering that 24-26% of the total nuclear DNA was matrix associated, these enrichments indicated that 80-93% of the hsp 70 genes were in fact matrix associated. As expected from this calculation, the data in Fig. 3E showed that the DNA cleaved from the matrix (supernatant DNA) which contains 76% of the total nuclear DNA was depleted for these sequences (lanes S_C and S_H). Thus, even though hsp 70 genes were transcribed by less than 1 of every 20 cells during normal growth conditions, these genes were matrix associated. As seen in

Fig. 3A, lane HS, the matrix association of the hsp 70 gene was unchanged by heat shock.

Nearly identical results were found for the hsp 23 gene, as seen in Fig. 3B. This gene was enriched 2.6-3.5 fold compared to total nuclear DNA in several matrix preparations made from control or heat shocked cells.

The actin gene was matrix associated during normal growth conditions (Fig. 3C, lane C). Heat shock, which disrupted this transcription, did not disrupt its matrix association (Fig. 3C, lane HS). The actin gene enrichment varied from 2.7 to 3.7 fold in 8 separate matrix DNA preparations. The large differences in actin sequence content between matrix associated and non-associated DNA can be seen in Fig. 1B (compare lane M to lane S).

The chorion gene, nontranscribed at either temperature, was depleted in the DNA associated with the matrix (Fig. 3D). Matrix DNA contained a chorion gene concentration only 25% that of total nuclear DNA. This depletion was not changed by heat shock (Fig. 3D, lane HS). Nearly identical depletions were observed in a 4.8 kb EcoRI fragment containing the alcohol dehydrogenase gene, another gene not transcribed in Kc cells under normal or heat shock conditions (data not shown).

The difference between matrix DNA content of active and inactive genes was further emphasized in Fig. 3F. For this figure, a filter containing 1 ug of total nuclear DNA and 1 ug of matrix DNA was prepared. When hybridized with both the actin and chorion gene probes simultaneously, the enrichment of the actin gene with concomitant depletion of the chorion gene was obvious.

Previous work has shown that hnRNAs are bound to the nuclear matrix (36-39). It was possible that genes were associated with the matrix through their association with this bound hnRNA. To test this possibility, matrix-halo structures were prepared as before, but half of them were digested with EcoRI plus RNAase A and half with EcoRI alone. Studies in which cells were first pulsed for 1 min. with ³H-uridine showed that while over 90% of nascent RNA remained attached to the nuclear matrices when digested with EcoRI alone, only 0.3% of the nascent RNA remained when the matrices had been digested with EcoRI plus 50 ug/ml RNAase A. Digestion with RNAase A had no effect on the percentage of total nuclear DNA remaining attached to the matrix. As shown in Fig. 3B and 3C, lanes C_R, matrices digested and EcoRI with RNAase A had equal enrichments for the hsp 23 and actin genes. When RNAse A was used to treat nuclei before

salt extraction as well as included during restriction endonuclease digestion, identical results were obtained. RNAase A also did not change the matrix association of the hsp 70 genes or the depletion of the chorion gene (data not shown). Hence, nascent RNA probably was not responsible for the attachment of genes to the nuclear matrix.

We next examined the actin gene matrix association in more detail. We specifically determined whether the whole actin gene or only some portion of it was bound to the matrix. First, we assessed whether the 8.7 kb EcoRI fragment containing the entire transcription unit of the cytoplasmic actin gene was the only fragment in this region enriched in the matrix preparations. To determine this, we used DmA2, a 17.5 kb phage clone, which contained the 8.7 kb EcoRI actin gene fragment as well as nontranscribed 5' and 3' EcoRI fragments surrounding it (32). Only the 8.7 kb fragment was enriched in matrix DNA (not shown). To further define the region of DNA mediating the binding of the 8.7 kb fragment to the matrix, we performed a double digestion experiment. Matrix-halo structures were prepared as usual but half were digested with EcoRI alone and half with both EcoRI and XbaI. This double digestion resulted in 21% of the total nuclear DNA remaining with the matrix in contrast to 24% remaining after EcoRI digestion alone. Three actin gene fragments were generated by EcoRI/XbaI double digestion: (1) a 3.5 kb fragment containing upstream sequences, a short leader and most of the intron; (2) a 3.0 kb fragment containing the majority of the transcribed region and, (3) a 2.2 kb fragment which contains only nontranscribed sequences (see Fig. 4D). When we compared total DNA to DNA from matrices which were digested with EcoRI alone, all three fragments were seen to be enriched (Fig. 4A). This was expected because it has already been shown (Fig. 3C) that the 8.7 kb EcoRI fragment was enriched in nuclear matrices prepared with EcoRI, and the further XbaI digestion of the purified matrix DNA just cleaved the enriched 8.7 kb fragment into three enriched pieces. However, in the matrix DNA generated by double digestion of the matrices with EcoRI and XbaI, only the 3.5 kb fragment at the 5' end of the gene was enriched. The 3.0 kb fragment which contains 70% of the transcribed sequences was not enriched and the 2.2 kb fragment was significantly depleted in the matrix DNA.

The hsp 70 genes were also examined to see if cleaving this gene internally would release some of the transcribed sequences from the matrix. This experiment was performed as noted above for actin, but the double digestion was instead carried out with EcoRI and PstI. The

EcoRI/PstI double digestion left 22% of the total nuclear DNA associated with the matrix. Four major fragments were detected with an hsp 70 structural gene probe. The 2.7, 2.0 and 1.3 kb fragments include more than 75% of the transcribed sequences of the hsp 70 genes (Fig. 4E). The 3.8 kb fragment includes the 5' end of the hsp 70 genes and adjacent upstream sequences located at chromosomal position 87A7. Sequences 5' to the hsp 70 genes at chromosomal position 87C1 were not detected with the probe used. Fig. 4B shows that most of the 2.7, 2.1, and 1.3 kb fragments were detached from the matrix by PstI digestion. In contrast, the 3.8 kb fragment remained associated with the matrix after EcoRI and PstI double digestion. Hence, the results with the hsp 70 gene were similar to those found with the actin gene. The patterns obtained in double digestion experiments were identical in normal and heat shocked cells.

The result in Fig. 4A indicated that sequences within a 3.5 kb fragment at the 5' end of the actin gene apparently mediated its matrix binding. There were two models of binding which could explain this result. First,

Fig. 4A: The content of fragments of the actin gene in matrix DNA. Matrix-halo structures from normally growing cells were cleaved with EcoRI (M_E) or EcoRI plus XbaI (M_{E+X}) and DNA from the pelleted matrices isolated and purified as described in Methods. Equal quantities of purified matrix DNA and various quantities of total nuclear DNA were cleaved with EcoRI plus XbaI, and, after electrophoresis through an agarose gel, transferred to nitrocellulose. The filter was hybridized with the ^{32}P -labeled cytoplasmic actin gene probe.

Fig. 4B: The content of fragments of the hsp70 genes in matrix DNA. Matrix-halo structures from normally growing cells were cleaved with EcoRI (lane M_E) or EcoRI plus PstI (lane M_{E+P}) and DNA from the pelleted matrices was isolated and purified as described in Methods. Equal quantities of purified matrix DNA and various quantities of total nuclear DNA were cleaved with EcoRI plus PstI, and, after electrophoresis through an agarose gel, transferred to nitrocellulose. The filter was hybridized with a ^{32}P -labeled hsp70 gene probe.

Fig. 4C: The content of small fragments of the actin gene in matrix DNA. Matrix-halo structures from normally growing cells were cleaved with EcoRI (lane M_E) or EcoRI plus HinfI (lane M_{E+H}) and DNA from the pelleted matrices was isolated and purified as described in Methods. Equal quantities of purified matrix DNA and various quantities of total nuclear DNA were cleaved with EcoRI plus HinfI and, after electrophoresis through an agarose gel, transferred to nitrocellulose. The filter was hybridized with a ^{32}P -labeled plasmid probe containing the 3.5 kb EcoRI/SalI fragment of the cytoplasmic actin gene. Note that only 8% of the total nuclear DNA remained associated with matrices digested with EcoRI plus HinfI.

Fig. 4D: Restriction map of the cytoplasmic actin gene. The transcription unit is boxed, with the exons shaded.
E:EcoRI; X:XbaI; S:SalI; H:HinfI

Fig. 4E: Restriction map of the 5 hsp 70 genes. The transcription unit is boxed. P:PstI; E:EcoRI.

the binding could be mediated by a single specific sequence within the 3.5 kb actin fragment. In this case, one could presumably isolate a very small restriction fragment which maintained its enrichment. The second possibility was that the attachment was due to multiple interactions within the 3.5 kb segment. In that case, the binding could be mediated by several interactions, each too weak by itself to maintain this matrix association, but together strong enough to anchor the 3.5 kb fragment. To distinguish between these possibilities, matrix halo structures were digested with enzymes that cleaved the actin gene relatively frequently. For example, EcoRI plus HinfI digestion resulted in only 8% of the total DNA remaining with the matrix. This double digestion cleaved the 3.5 kb EcoRI/XbaI actin gene fragment four times, yielding fragments of 1.5, 1.1, a doublet of 0.66/0.67, and 0.4 kb. Each of these fragments were enriched in matrices digested with EcoRI alone. In contrast, each of these five fragments was released from matrices by EcoRI plus HinfI digestion of matrix-halo structures. For example, the 1.5 kb fragment was enriched 2.9 fold in the EcoRI digested matrices (lane ME in Fig. 4C). Since the EcoRI digested matrices in this preparation contained 25% of the total nuclear DNA, 73% ($2.9 \times 25\%$) of the total nuclear 1.5 kb actin fragment was matrix associated. After EcoRI plus HinfI digestion, the 1.5 kb fragment was 2 fold enriched. However, the EcoRI plus HinfI digested matrices contained only 8% of the total nuclear DNA, and therefore only 16% ($2.0 \times 8\%$) of the 1.5 kb fragment remained. Hence 77% [$(73-16)/73$] of the 1.5 kb actin fragment present in EcoRI digested matrices was released by HinfI treatment. Similar calculations showed that 80%, 89% and 94% of the 1.1, 0.66/0.67, and 0.4 kb fragments, respectively, were released by HinfI digestion. Digestion with EcoRI plus HhaI or EcoRI plus RsaI also showed that 80-90% of the matrix associated 3.5 kb EcoRI/XbaI generated actin fragment could be released by digestion with HhaI or RsaI. These results suggested that the anchorage of the 3.5 kb actin fragment to the matrix was mediated by multiple binding sites, any one of which was not stable enough to anchor the DNA subfragment to which it was attached under the conditions used.

DISCUSSION

The results of the experiments reported here lead to several conclusions. First, transcriptionally active genes in *Drosophila* were associated with the matrix while inactive genes were not. This supported

other experiments showing the enrichment of active genes in the nuclear matrices of avian and mammalian cells (12,13,15,17). Second, this association was not dependent on high rates of transcription of these genes. Genes which were being transcribed in less than 1 of 20 cells at the time of matrix preparation were as quantitatively associated with the matrix as genes which were abundantly transcribed. Third, this association was probably not mediated by nascent RNA. Fourth, a specific region of the actin gene (a 3.5 kb fragment encompassing 30% of the transcribed sequences at the 5' end of the gene) appeared to contain the major attachment sites of this gene to the nuclear matrix. These attachments were apparently mediated by more than one sequence within the 3.5 kb fragment, because when this fragment was further cleaved, the resultant subfragments were released from the matrix.

This last result is particularly important in explaining some of the contradictory results that have so far been found in analyses of matrix DNA. When digestion of the matrix-halo structures is limited so that fragments of DNA are produced which are on the order of a few kb or larger, one can reproducibly demonstrate that active genes within these fragments are matrix associated. However, it appears to require multiple interactions to anchor these fragments (Fig. 4). Hence, if the matrix associated DNA is cut into small pieces, either with frequently cutting restriction enzymes as reported here, or with nonspecific endonucleases as reported in previous studies (45,46), the matrix association of these active genes is not maintained. Our results may also be useful in explaining the data recently reported by Rose and Garrard (47). Using a very different procedure for generating matrices (micrococcal nuclease digestion in low salt buffer followed by EDTA treatment) these workers found that active immunoglobulin genes were enriched in matrices after low extents of digestion with micrococcal nuclease but not after extensive digestions. This result is consistent with the hypothesis that the matrix association of active genes requires multiple interactions for stability.

Another point worth mentioning is that the association of active genes with the matrix of *Drosophila* cells was not dependent on a high transcription rate. These results therefore differ in some ways from those reported with the chick oviduct (16,17). In that system withdrawal of hormonal therapy led to elimination of transcription of the ovalbumin gene with concomitant detachment of the gene from the matrix. The very different time courses of the two systems -- days in the case of the

oviduct and minutes in the case of heat shocked Drosophila cells -- may explain this difference. Regardless of the reason for these differences, however, it is clear from the Drosophila studies that the association of "active" genes with the nuclear matrix is in some cases independent of the mechanical act of transcription per se.

Several experiments suggest that transcriptional complexes are not mediating the association of active genes with the matrix. This is an important point, since supercoiled loops in procaryotes are apparently organized by such complexes (48-52). Unlike the situation in procaryotes, however, the present results show that exhaustive digestion of nascent RNA does not alter the configuration of genes with respect to the matrix. In addition, we have shown that genes associated with active RNA polymerases in only a fraction of the cells are associated with the matrix as quantitatively as very actively transcribed genes. Hence, high rates of transcription are not required for the matrix association. Finally, the results show that DNA fragments associated with the highest density of RNA polymerase II molecules known in eucaryotes (over 15 RNA polymerase II molecules per hsp gene during heat shock) are not enriched in matrices prepared with EcoRI and PstI digestion. This last result is inconsistent with the idea that RNA polymerases or associated factors are primarily responsible for the active gene-matrix associations noted in this and other studies.

During the review of this manuscript a paper appeared by Mirkovitch et al. (53) demonstrating the attachment of certain sequences to nuclear skeletal structures made from Drosophila cells. The conditions used to prepare these structures included the use of digitonin and EDTA, requisite exposure to copper or incubation at 37°C, and extraction with lithium diiodosalicylate. Despite these drastic differences, both the procedures used by us and other workers and those used by Mirkovitch et al. generate structures in which restriction fragments within or near actively transcribed genes appear to be matrix bound. In particular, a heat shock protein fragment near the 5' end of the heat shock genes at chromosomal position 87A7 were bound to both types of nuclear structures. However, there were two major differences between the results reported by Mirkovitch et al. and the results reported herein. First, in some experiments they used high salt to prepare skeletal structures and their results with high salt were quite different than ours. This difference is probably explained by the different high salt extraction techniques used by Mirkovitch et al.

and us, including Mirkovitch's use of EDTA during the high salt treatment. We have found that magnesium is crucial for the stability of the matrix structure in high salt. The second important difference between our work and theirs is that they could find attachment points localized to relatively small fragments (several hundred base pairs) while we found that the attachment of larger fragments (3-5 kb) was apparently mediated by multiple interactions, each not stable enough by itself to maintain its individual binding when isolated in a small fragment. It is possible that the conditions used by Mirkovitch *et al.* stabilize the same attachments found in high salt, or alternatively that the attachment points they find are completely different or are generated by their conditions. It is important to note that the small fragments bound under their conditions did not fractionate as expected on the basis of a DNA loop model. Cleavage of the DNA in matrix-halo structures with restriction enzymes that recognize 4 bp sequences should drastically reduce the amount of DNA left bound to the matrix. Fragments remaining attached to the matrix should be much more highly enriched after such cleavage than after cleavage with restriction enzymes that cleave infrequently in DNA (see our discussion of the results shown in Fig. 4). In the results reported by Mirkovitch *et al.*, 30% of the total DNA apparently remained attached to the structures regardless of whether DNA was cut infrequently (with EcoRI or HindIII) or frequently (MboI or HinfI plus EcoRI and HindIII). Furthermore, no greater enrichment of fragments (compared to total nuclear DNA) was seen when MboI or HinfI was used than when EcoRI or HindIII was used. It is difficult to reconcile such results with a loop model for attachment points.

The results reported here show that the matrix association of active genes in *Drosophila* has certain features in common with other aspects of chromatin structure. For example, the matrix association of hsp 70 genes exists both before and during heat shock as do hypersensitive sites in chromatin (23,24). This is unlike other chromatin structural features. For example, general nuclease sensitivity as assessed with DNAase I or micrococcal nuclease digestion of active genes drastically changes during heat shock (22,23). In addition, it appears to require more than one specific sequence to fully anchor the actin gene to the matrix. Data from other laboratories have shown that there is usually more than one hypersensitive site surrounding a transcriptionally active gene (22,54-59). It might be speculated that some of the proteins responsible for DNAase I hypersensitivity are also responsible for the matrix association of active genes. In order to prove this speculation, much

further work, on both the proteins mediating DNAase I hypersensitivity and those mediating matrix association, will be needed.

ACKNOWLEDGEMENTS

The authors thank J. Lis, E. Fyrberg, and A. Spradling for providing cloned DNA sequences, and J. Lis, D. Gilmour, B. Zehnbauser and S. Goelz for helpful discussions. This work was supported by training grants GM07309 and CA-09243, and grant GM31676 awarded by the NIH, DHHS.

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REFERENCES

1. Weisbrod, S. (1982). *Nature* **297**:289-295.
2. Cartwright, I.L., Abmayr, S.M., Fleischmann, G., Lowenhaupt, K., Elgin, S.C.R., Keene, M.A., and Howard, G.C. (1982). *CRC Crit. Rev. Biochem.* **13**:1-85.
3. Berezney, R., and Coffey, D.S. (1977). *J. Cell Biol.* **73**:616-637.
4. Comings, D.E. and Okada, S.A. (1976) *Exp. Cell Res.* **103**:341-360.
5. Capco, D.G., Wan, K.M., and Penman, S. (1982). *Cell* **29**:847-858.
6. Kaufmann, S.H., Coffey, D.S., and Shaper, J.H. (1981). *Exp. Cell Res.* **132**:105-123.
7. Bouteille, M., Bouvier, D., and Seve, A.P. (1983). *Int. Rev. Cyt.* **83**:134-182.
8. Cook, P.R. and Brazell, I. A. (1975) *J. Cell Sci.* **19**:261-279.
9. Benyajati, C. and Worcel, A. (1976). *Cell* **9**:393-407.
10. Vogelstein, B., Pardoll, D., and Coffey, D.S. (1980). *Cell* **21**:79-85.
11. Lebkowski, J.S. and Laemmli, U.K. (1982). *J. Mol. Biol.* **156**:309-324.
12. Nelkin, B., Pardoll, D., and Vogelstein, B. (1980). *Nucl. Acids Res.* **8**:5623-5638.
13. Jackson, D.A., McCreedy, S.J., and Cook, P.R. (1981). *Nature* **292**:552-555.
14. Cook, P.R., Long, J., Hayday, A., Lanin, L., Fried, M., Chiswell, D.J., and Wyle, J.A. (1982). *EMBO J* **1**:447-452.
15. Robinson, S.I., Nelkin, B.D., and Vogelstein, B. (1982). *Cell* **28**:99-106.
16. Robinson, S.I., Small, D., Idzerda, R., McKnight, G.S., and Vogelstein, B. (1983). *Nucl. Acids Res.* **11**:5113-5130.
17. Ciejek, E.M., Tsai, M.J., and O'Malley, B.W. (1983). *Nature* **306**:607-609.
18. Ashburner, M. and Bonner, J.J. (1979). *Cell* **17**:241-254.
19. Findly, R.C. and Pederson, T. (1981). *J. Cell Biol.* **88**:323-328.
20. Greenleaf, A.L., Plagens, U., Jamrich, M., and Bautz, E.K.F. (1978). *Chromasoma (Berl.)* **65**:127-136.
21. Sass, H. (1982). *Cell* **28**:269-279.
22. Levy, A. and Noll, M. (1981). *Nature* **289**:198-203.
23. Wu, C., Wong, Y.C., and Elgin, S.C.R. (1979). *Cell* **16**:807-814.
24. Wu, C. (1980). *Nature* **286**:854-860.
25. Echallier, G. and Ohanessian, A. (1969). *C. R. Acad. Sci. (Paris)* **268**:1771-1773.
26. Echallier, G. and Ohanessian, A. (1970). *In Vitro* **6**:162-172.
27. Weber, J., Jelinek, W., and Darnell, J.E., Jr. (1977). *Cell* **10**:611-616.

-
28. McKnight, G.S. and Palmiter, R.D. (1979). *J. Biol. Chem.* **254**:9050-9058.
 29. Groudine, M., Peretz, M., and Weintraub, H. (1981). *Mol. Cell. Biol.* **1**:281-288.
 30. Lis, J.T., Neckameyer, W., Dubensky, R., and Costlow, N. (1981). *Gene* **15**:67-80.
 31. Spradling, A.C., Digan, M.E., Mahowald, A.P., Scott, M. and Craig, E.A. (1980). *Cell* **19**:905-914.
 32. Fyrberg, E.A., Kindle, K.L., Davidson, N. and Sodja, A. (1980). *Cell* **19**:365-378.
 33. Vogelstein, B., Nelkin, B., Pardoll, D., Robinson, S., and Small, D. (1982). *In Gene Regulation* (O'Malley, B., ed.) Academic, NY, pp. 105-119.
 34. Buongiorno-Nardelli, M., Michele, G., Carrie, M.T., and Marilley, M. (1982). *Nature* **298**:100-103.
 35. Peacock, W.J., Brutlag, D., Goldrin, E., Appels, R., Hinton, C.W., and Lindsley, D.L. (1973). *Cold S. Harb. Symp. Quant. Biol.* **38**:405-416.
 36. Miller, T.E., Huang, C.-Y., and Pogo, A.O. (1978). *J. Cell Biol.* **76**:675-691.
 37. Herman, R., Weymouth, L., and Penman, S. (1978). *J. Cell Biol.* **78**:663-674.
 38. Van Eekelen, C.A.G. and Van Venrooij, W.J. (1981). *J. Cell Biol.* **88**:554-563.
 39. Clejek, E.M., Nordstrom, J.L., Tsai, M.J., and O'Malley, B.W. (1982). *Biochem.* **21**:4945-4953.
 40. Brown, A. J. L. and Ish-Horowicz, D. (1981) *Nature* **290**:677-682.
 41. Ingolia, T.D., Craig, E.A., and McCarthy, B.J. (1980). *Cell* **21**:669-679.
 42. Moran, L., Mirault, M.E., Tissieres, A., Schedl, P., Artavanis-Tsakonas, S., and Gehring, W. J. (1979). *Cell* **17**:1-8.
 43. Mirault, M.E., Goldschmidt-Clermont, M.; Artavanis-Tsakonas, S., and Schedl, P. (1979). *Proc. Natl. Acad. Sci. (USA)* **76**:5254-5258.
 44. Goldschmidt-Clermont, M. (1980). *Nucl. Acids Res.* **8**:235-252.
 45. Pardoll, D. and Vogelstein, B. (1980) *Exp. Cell Res.* **128**:466-470.
 46. Basler, J., Hastie, N.D., Pietras, D., Matsui, S.I., Sandberg, A.A., and Berezney, R. (1981). *Biochem.* **20**:6921-6929.
 47. Rose, S.M., and Garrard, W.T. (1984). *J. Biol. Chem.* **259**:8534-8544.
 48. Pettijohn, D.E. and Hecht, R. (1973). *Cold S. Harb. Symp. Quant. Biol.* **38**:31-42.
 49. Worcel, A., Burgi, Jr., E., Robinton, J., and Carlson, C.L. (1973). *Cold S. Harb. Symp. Quant. Biol.* **38**:43-32.
 50. Worcel, A. and Burgi, E. (1974). *J. Mol. Biol.* **82**:91-105.
 51. Stonington, O.G., and Pettijohn, D.E. (1971). *Proc. Natl. Acad. Sci. (USA)* **68**:6-9.
 52. Dworsky, P., and Schaechter, M. (1979). *J. Bact.* **116**:1364-1374.
 53. Mirkovitch, J., Mirault, M.-E., and Laemmli, U.K. (1984). *Cell* **39**:223-232.
 54. Wu, C., Bingham, P.M., Livak, K.J., Holmgren, R., and Elgin, S.C.R. (1979). *Cell* **16**:797-806.
 55. Keene, M.A., Corces, V., Lowenhaupt, K., Elgin, S.C.R. (1981). *Proc. Natl. Acad. Sci. (USA)* **78**:143-146.
 56. Costlow, N. and Lis, J.T. (1984). *Mol. Cell Biol.* **4**, in press.
 57. Wong, K.S., O'Connell, P., Rosbash, M., and Elgin, S.C.R. (1981). *Nucl. Acids Res.* **9**:6749.
 58. Stadler, J., Larsen, A., Engel, J.D., Dolan, M., Groudine, M., and Weintraub, H. (1980). *Cell* **20**:451-460.
 59. Weintraub, A., Larsen, A., and Groudine, M. (1981). *Cell* **24**:333-344.
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