amide gives optimal contrast. Under these conditions R-loops are less stable; part of the RNA may be displaced by branch migration, which leads to the appearance of RNA tails or whiskers. Correct interpretation of RNA tails may be of the greatest importance. In principle, RNA tails can be interpreted in two ways: either they are an artefact produced by displacement of the RNA, e.g., in A + T rich regions,[132] or the RNA molecule does not hybridize completely, because part of the homologous sequence is not present in the DNA molecule studied. This question has been discussed by Chow et al.,[131] who mapped the early and late RNA transcripts from adenovirus 2-infected cells by the R-loop method. They observed frequent RNA tails, both at the 3′ and 5′ ends of many R-loops, which were interpreted to be the result of displacement of the RNA by the second DNA strand. The frequency of tails could be decreased by spreading the sample within seconds after dilution into the spreading mixture. Even then, a large proportion of R-loops had short tails at their 5′ ends. Similar observations were made by Westphal et al.[134] and Meyer et al.[135] As later studies have shown, the 5′ end leader sequences of adenovirus mRNAs are encoded elsewhere in the genome, and thus the 5′ tails are real structures of biological importance.[123-126,136] Another example was presented by Tonegawa et al.[119] and Brack et al.[25] in their R-loop analysis of immunoglobulin genes. In embryonic cells, the variable and constant region of the light-chain genes are on separate DNA fragments. R-loops on these molecules were only half the expected size; the other half of the mRNA was extended as a tail.

Branch migration may be reduced by spreading the R-loop molecules from 70% formamide solutions,[23,25,132] but this may result in poorer contrast and lower quality of the cytochrome film. Also, A + T rich sequences may not fully base pair under these conditions.

To overcome these problems, Kaback et al. have devised a method for stabilizing the R-loops by modifying the free DNA single-strand in the R-loop with glyoxal. At temperatures considerably below the Tm of DNA, and under suitable selective reaction conditions, glyoxal specifically modifies G residues in single-stranded DNA.[99] When R-loop molecules were treated with 1.0 *M* glyoxal at 12°C (heating in glyoxal above 12°C results in denaturation of duplex DNA), they could be spread from 50% formamide, and no shortening of the R-loops due to branch migration was observed. In addition, some small R-loops that had been selectively lost during 50% formamide spreading (compared to the 70% formamide spreading) could be stabilized by the glyoxal treatment. Once fixed with glyoxal, R-loops are completely stable; they can be dialyzed or purified by gel filtration and stored for weeks without major degradation.[132] Combining both Trioxsalen cross-linking of the DNA before R-loop formation with glyoxal fixation afterwards, Kaback et al.[132] were able to study the distribution of R-loops made with total poly A⁺ yeast RNA on total yeast DNA. By this method they were able to calculate the number of genes transcribed into poly A⁺ mRNA per yeast genome. One problem was the interpretation of very short loops (less than 200 base pairs long). It is difficult to determine whether these are very short R-loops or denaturation bubbles introduced by the glyoxal treatment.

D. Orientation of R-Loops

Certain problems may be encountered with the orientation of genes visualized with the R-loop method. In cases where R-loops are made with poly A containing messengers, it may be possible to see short RNA tails extending from R-loops, corresponding to the poly A tails at the 3′ end of the mRNA[25,137] (see Figure 14). Such tails are not always visible, either because they are too short or because they are not sufficiently extended by the spreading procedure.

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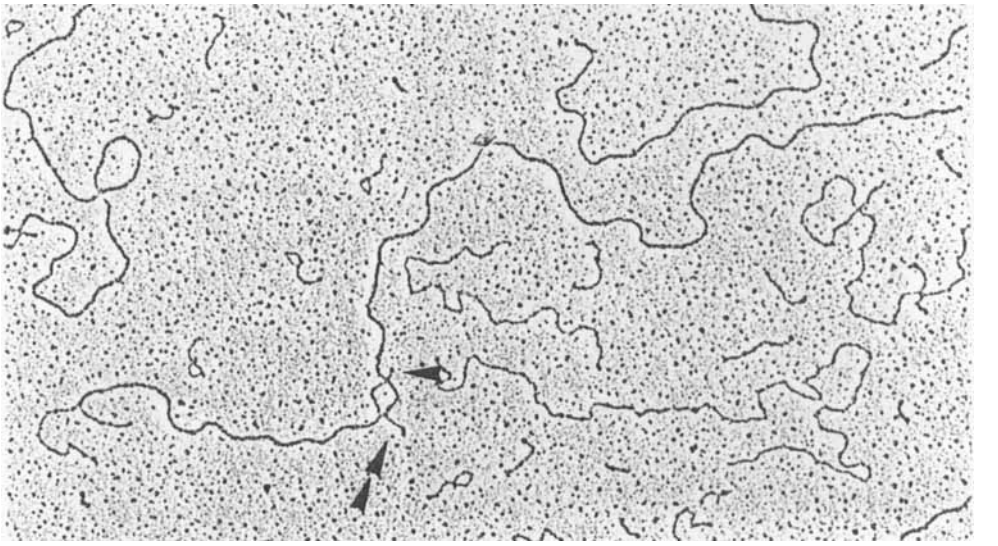

FIGURE 14. R-hybrid molecule.[131] Two mouse immunoglobulin gene clones — one containing the embryonic Cκ gene,[99] the second containing a myeloma Cκ + Vκ (MOPC 21) gene — were hybridized in the presence of a κ (MOPC 321) mRNA. The branch point f the heteroduplex indicates the position of the Cκ gene. Note also the RNA tails on the R-loop — the longer one corresponding to nonhybridized Vκ321 sequences (→→), the shorter one corresponding to the 3′ end poly A tail (→).

Bender and Davidson devised an elegant method for mapping poly(A) tails on RNA molecules by hybridizing them to poly(dT) tailed circular DNA molecules.[138] They observed that terminal transferase can polymerize poly(dT) tails at nicks in nicked circular molecules. It is best to use small circular duplex molecules like SV40 or minicircles of trypanosome kinetoplast DNA, because they are easily distinguishable in the electron microscope. These tailed molecules have been hybridized to poly A tails of oncornavirus RNA molecules[138] or to mRNA poly A tails in R-loops.[139,140,193] Broker et al.[125] and Chow et al.[123] have hybridized isolated single strands of restriction fragments containing the tripartite leader sequences to mark the 5′ end tails in R-loops on adenovirus DNA.

E. R-Hybrids

When two partially homologous DNA molecules are to be compared by heteroduplex analysis, it may be useful to have an internal gene marker for the orientation of the hybrid molecules as well as for more accurate measurement of the position of branch points in long molecules. We have combined the heteroduplex and R-loop methods to form so-called "R-hybrids", which are heteroduplex molecules containing R-loops.[131] The conditions are similar to the ones described above for the two-step R-loop formation. The two different DNA fragments are mixed in equimolar amounts in the R-loop buffer in the presence of mRNA. The samples are heated to 10°C above the Tss, then incubated in decreasing temperature. The R-hybrid in Figure 14 shows the relationship between two immunoglobulin DNA clones; the R-loop maps the position of the constant region gene.

This method can be very useful for the comparison of related DNA clones of a gene family. As an example, different immunoglobulin gene clones containing variable region genes of one κ group with varying degrees of homology to a given κ mRNA have been analyzed (Figure 15). The position of the R-loop (i.e., V gene) allows accurate

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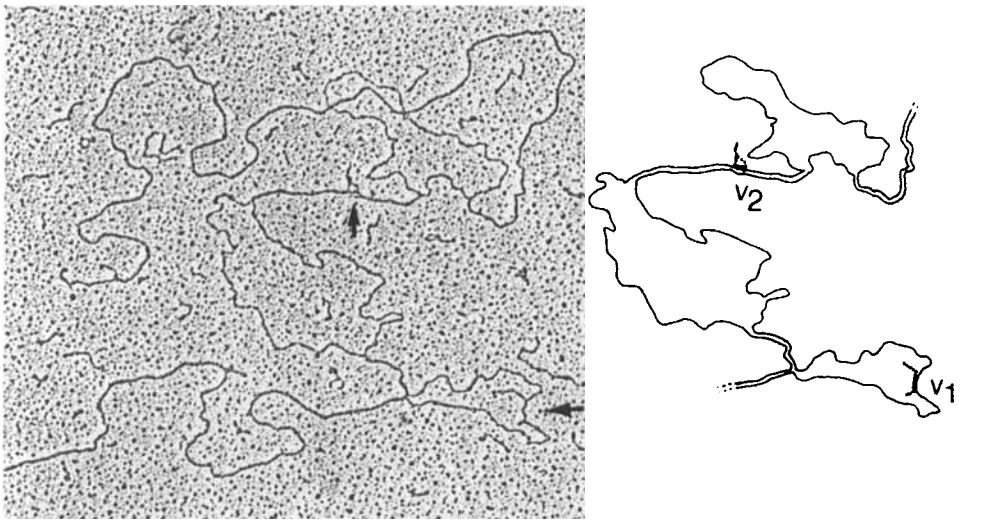

FIGURE 15. R-hybrid molecule showing the sequence relation between two partially homologous mouse immunoglobulin κ chain clones. The two DNAs (phage λgt vector, including the cloned fragments) were hybridized in the presence of κ mRNA, as described in the text. One DNA clone contains two copies of a κ variable region gene labeled with the mRNA (→); the second DNA contains one copy of a variable region gene that is surrounded by a region of homology.[131]

mapping of the very short homology regions within the rather complex hybrid structures. It could be shown that a region of about 1.5 to 2 kb flanking the variable region genes was conserved in eight related clones, whereas other regions have diverged to different degrees.[131]

F. Mapping of Short Hybrid Regions and tRNA Genes

The protein monolayer technique imposes some limitations on the resolution of very small R-loops on duplex DNA or short RNA:DNA hybrid regions along a single-stranded DNA. The shortest R-loops measurable as a loop structure are about 100 nucleotides long; short RNA:DNA hybrids on single-stranded molecules are even more difficult to detect and measure. Exceptions are the short homology regions in intron-exon structures, e.g., the hinge region or J regions in immunoglobulin genes (both about 39 nucleotides long[25,122,131]), detection and mapping of which was made possible because the mRNA also hybridized to other longer regions on the same DNA molecule, drawing the intron between the two coding regions into a characteristic loop.

There are two ways to increase the resolution of the method: (1) increase the resolution of the spreading method, and (2) increase the visibility of the target by tagging it to an electron dense particle or easily recognizable structure, so that it can be spread with conventional methods.

Wu and Davidson[58] developed a method called "gene-32 protein staining" which permitted visualization and mapping of several tRNA genes as well as 5S, 23S, and 16S ribosomal genes on the DNA of a transducing phage. This method involves binding of the T4 gene-32 protein to the single-stranded parts of a hybrid molecule and spreading these complexes with the ethidium bromide technique of Koller et al.[57] The hybrids were dialyzed against 0.01 *M* phosphate buffer, pH 6.8. After addition of gene-32 protein, the samples were incubated at 37°C, and the complexes were then fixed with glutaraldehyde. Ethidium bromide was added and the complexes were mounted on carbon-coated collodion films as described by Koller et al.[57] The distinction between duplex regions (thickness ~3.5 nm) and protein-coated single-stranded

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regions (~ 8 nm) was quite clear, and the short duplex regions of 85 ± 10 nucleotides corresponding to the tRNA genes could be localized.

Other spreading methods promising higher resolution, such as direct mounting on charged carbon films or BAC spreading, have either not been tried so far or have not given any improvements. The second approach — namely, tagging of specific electron opaque markers (ferritin, plastic spheres, specific antibodies) to tRNA molecules — will be described in a separate section.

G. Application and Value of the R-Loop Method

The impact of the R-loop method on recent developments in molecular biology has been enormous. It is at present one of the most important electron microscope techniques for the analysis of gene structure and genome organization. R-loop mapping has contributed to the discovery of intervening sequences and the mosaic nature of eukaryotic genes. In their original paper, White and Hogness discovered that when R-loops were formed with *Drosophila melanogaster* rRNA and DNA, the 28S genes were interrupted by intervening sequences and formed the characteristic triple-loop structure.[113] Soon afterwards, similar intron-exon structures were observed in cloned immunoglobulin genes[137] (Figure 16) and β-globin genes.[141] The complex architecture of the adenovirus genome was discovered, and the splicing pathways of early and late adenovirus mRNAs worked out mainly with the R-loop technique.[123-127]

R-loop analysis has shown the presence of a very short gene segment J which plays an important role in the somatic recombination of immunoglobulin genes.[25,131] The correlation between coding regions (R-loops) and protein domains in immunoglobulin heavy chains was suggested by electron microscopic R-loop mapping before it was confirmed by DNA sequencing.[122,139]

It should also be possible to apply the R-loop method to the analysis of gene function. As discussed above, it is now possible to examine the distribution of R-loops on large genome fractions. This should permit analysis of the expression of certain genes at different periods of differentiation. Another application of the R-loop method to study in vitro transcription and promoter mapping will be discussed in Section IX.

VII. LIMITING FACTORS IN HYBRIDIZATION METHODS

What is the minimum hybrid length detectable by electron microscopic methods? How perfect is the homology between two sequences that form a double-stranded hybrid, or how many mismatched bases can be accommodated in a hybrid before one starts to see single-stranded nonhomology regions? These and similar questions often arise when the limitations of hybridization methods are discussed.

It is very difficult to answer these questions because at present we do not know to what extent different hybridization methods (liquid hybridization, filter hybridization, and electron microscope conditions) give comparable results and how far theoretical data or model experiments with other methods are applicable to structures observed in the electron microscope. As we have discussed earlier, the resolution of spreading methods no longer limits the visualization of short hybrids: tagging methods,[142,143] gene-32 protein mounting,[58] or observation of intron-exon loops[25,122,123] and underwound loops[103] allow detection of very short homology regions. In the following we try to compare some fundamental data obtained by biochemical methods with electron microscope observation of short hybrid regions, the nucleotide sequences of which have been determined.

A number of parameters controlling specific hybridization processes have been defined (see References 115, 144 and references therein). These are (1) the minimum

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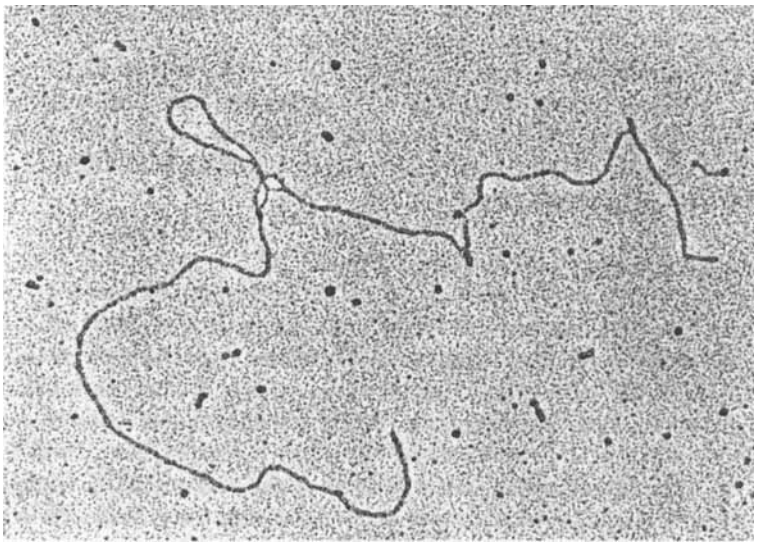

FIGURE 16. Triple R-loop or intron-exon loop molecule of an immunoglobulin κ light chain gene. The clone Ig25κ was hybridized with MOPC 321 mRNA. An intervening sequence (intron) loop of 2.9 kb separates the variable and the constant region gene. (From Lenhard-Schuller, R., Hohn, B., Brack, C., Hirama, M., and Tonegawa, S., *Proc. Natl. Acad. Sci. U.S.A.*, 75, 4709, 1978.)

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number of interacting nucleotides (minimal stable length); (2) the composition of interacting nucleotides (G + C content); (3) the number, type and location of mismatched bases (degree of complementarity); and (4) the presence or absence of adjacent occupancy. Futhermore, all hybridization reactions depend on the experimental conditions: ionic strength, temperature, DNA concentration, etc.

A. Minimum Stable Length

Niyogi and Thomas studied the influence of chain length and base composition on the specific association of oligoribonucleotides with denatured DNA.[144,145] Oligoribonucleotides of various chain lengths synthesized in vitro on T2, T4, T3, and T7 phage DNAs were annealed to denatured template DNA immobilized on membrane filters. The reactions were carried out in 5 × SSC. In the absence of Mg^{++}, a chain length of ten or more ribonucleotides was required to form an RNase resistant complex, whereas in the presence of 0.01 *M* Mg^{++}, octanucleotides could form detectable hybrids. Even the shortest oligonucleotides capable of forming stable hybrids did so with a very high degree of specificity; discrimination between T-even and T-odd phage DNAs occurred when passing from a length of nine to ten nucleotides.[143] The minimal length for interacting synthetic polynucleotides is about four to five.[115]

The shortest DNA:DNA hybrids observed in the electron microscope approached these values. Under certain conditions the sticky ends of λ DNA (12 nucleotides) can pair and circularized molecules are stable enough to be spread in cytochrome monolayers. We have even observed circularization of DNA fragments that had been cleaved with the restriction endonuclease *HindIII*. (Staggered cleavage of double-stranded DNA with this enzyme creates sticky ends of four bases.) When PM2 *HindIII* fragment 2 (~2.2 kb long) was adsorbed on pentylamine-charged carbon films in the presence of 20μg/m*l* ethidium bromide, more than 50% of the molecules appeared as small circles.[131] Apparently, the intercalated ethidium bromide confers to the DNA fragments the flexibility (or rigidity or, perhaps, the extra length) needed for circulariza-

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tion. Pairing of the four nucleotides AGCT is stable enough to withstand the adsorption procedure. It was also with the direct mounting procedure involving gene-32 protein and ethidium bromide that Wu and Davidson observed very short, inverted repeat, snap-back structures with a stem of 15 ± 7 nucleotides[58] or short, secondary structure loops on polyoma DNA with stems of 30 to 40 nucleotides.[109] Loops and stems due to the short, inverted repeat sequences flanking the *E. coli* 16S and 23S rRNA genes were first observed with the gene-32 protein method.[58] The length of the stems is 26 and 29 base pairs, respectively (see References.[109])

The shortest RNA:DNA hybrids that have been observed in formamide-cytochrome c spreadings of R-loops or single-stranded DNA:RNA hybrids are about 40 base pairs long. These are the J sequences of immunoglobulin λ and κ chains,[25,131] the hinge region in γl heavy chain,[122] and the leader sequences of adenovirus-2 mRNAs.[123,136] The nucleotide sequences of these short coding regions have been determined. J segments and the hinge region are 39 nucleotides long,[122,146,147] and the leader segments of adenovirus are 42, 71, and 89 nucleotides long.[148] These short sequences formed stable hybrids with a high degree of specificity (see next section).

It must be pointed out that all these very short hybrid regions observed in electron microscope preparations were created by intramolecular hybridization: snap-back structures on single-stranded DNA, annealing of two sticky ends, and hybridization of RNA molecules that had been stabilized by additional longer homology regions on the same DNA molecule (intron-exon loops). Intramolecular hybridization is a first-order process, and the conditions both for formation and stabilization of these hybrids are obviously more favorable than the ones for intermolecular hybridization, a second order process.

B. Degree of Homology

The degree of specificity attainable in nucleic acid hybridization is open to experimental manipulation.[115] One can choose conditions according to what kind of homology is to be looked for: under very stringent conditions only perfectly matched duplexes are formed, whereas under less stringent conditions heterologous DNAs with higher numbers of mismatched bases can form hybrids. It is thus possible to study the degree of sequence homology between two related DNA molecules.[24]

The degree of sequence homology refers to the fraction of bases in a given DNA segment that are mismatched and therefore cannot base pair.[1] It is reflected in the melting temperature of the DNA segment, as unpaired bases destabilize the helix structure. As Ferguson and Davis[1] point out, it is very important to carefully control the effective temperature (i.e., the temperature relative to the melting temperature of the DNA under a given set of conditions) of the DNA molecule during preparation procedures. One can achieve any given effective temperature by a variety of conditions (changing ionic strength, formamide concentration, temperature).

The spreading conditions most frequently used for mounting heteroduplex molecules are Tm −25°C.[1,9,149] Assuming that every 1% base mismatch lowers the Tm by 0.7°C,[115] a DNA duplex containing up to 35% mismatch would still appear as a perfect duplex in the electron microscope. When the isodenaturing spreading procedure is employed,[24] it is possible to control the effective temperature. This was illustrated in a study of the sequence relationship between T7 and T3 phage DNAs.[24] Heteroduplex molecules between T7 and T3 DNA were mounted under different isodenaturing conditions (i.e., different effective temperatures). With increasing effective temperature the fraction of duplex regions decreased. A percent sequence homology map of the two genomes could be constructed and compared to the genetic map of the two related phages. The sequence relationship between two *Bacillus subtilis* phage DNAs has been established with similar methods.[150]

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The specificity of RNA:DNA hybridization under R-loop conditions can be illustrated with the example of the immunoglobulin Jκ sequences. Electron microscopic R-loop mapping has allowed the detection of four Jκ sequences that are clustered close to the κ constant region gene on embryonic mouse DNA.[131] They were identified by different κ mRNAs each hybridizing to a specific J sequence. Only very little cross hybridization was observed. Determination of the nucleotide sequence of this region has confirmed the presence of five specific J sequences[147,151] and shown the sequence relation between them. It is now possible to compare exactly the degree of homology between given pairs of J sequences and the specificity of R-loop formation of homologous or heterologous regions. For example, the minimum difference between J1 and J2, or J2 and J4, is a mismatch of 6 out of 39 bases, and the maximum (J1 and J3 or J3 and J4) is a mismatch of 11 out of 39 bases. In the first case, about 10 to 20% cross hybridization could be observed. In all other cases with 8 to 11 mismatched bases, no cross hybridization occurred. The R-loop hybridization conditions (high salt, high temperature, close to Tss) are very stringent and permit discrimination between these very short related J sequences. It is surprising that specific hybridization to a Jμ sequence that is separated from the constant region of the μ heavy chain gene by 6 to 7 kb could be detected with these methods.[152]

VIII. TAGGING METHODS

The limitations imposed by the protein monolayer technique on the resolution of small hybrid regions along a DNA molecule or small proteins bound to nucleic acids have prompted several groups to develop methods for indirect visualization of these structures. These "tagging" methods are based on the following strategy: an electron dense label is tagged directly or indirectly to the target site; these labeled molecules can then be mounted by conventional spreading methods.

A. Direct Labeling with Ferritin

In the first attempt at mapping 4S genes and tRNA genes, the electron dense protein ferritin has been coupled directly to the 3′ end of 4S RNA or tRNA molecules.[142,153] These labeled RNA molecules were hybridized to the DNA strand or heteroduplex molecule of interest. The position of the ferritin molecules on the DNA molecule indicates the position of the hybrid and allows mapping of the corresponding genes.

Several procedures have been described for the covalent coupling of ferritin to RNA molecules.[142,153] In all methods, the 3′ end of the RNAs is oxidized to the dialdehyde with periodate. Ferritin is acetylated with bromoacetic acid anhydride. The oxidized RNA is coupled through Schiff base formation to an amino group of cystamine, followed by reduction with sodium borohydride and dithiothreitol, or coupled to mercap tosuccinic acid dihydrazide. Reaction of this RNA-SH with the bromoacetylated ferritin gives rise to the RNA-ferritin conjugate. Alternatively, oxidized RNA is coupled directly to one of the amino groups of unmodified ferritin, and the product is stabilized by borohydride reduction. Uncoupled RNA can be separated from the RNA-ferritin complex on a sucrose gradient.[142] Since a large excess of ferritin-tRNA is needed to obtain effective hybridization to tRNA genes, unhybridized ferritin-tRNA must be removed after the hybridization, either by phenol extraction or by gel electrophoresis; otherwise, the background of ferritin label in the spread sample is too high. This tagging method has enabled the mapping of nine 4S genes on the heavy strand of HeLa cell mitochrondrial DNA,[153] and of several tRNA genes on heteroduplex molecules of transducing phages.[142]

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B. Ferritin-Avidin:Biotin Labels

An alternate method for attaching ferritin to RNA in short RNA:DNA hybrids was described by Angerer et al.[143] and Broker et al.[154] It is based on the high affinity interaction of the egg-white protein avidin with biotin. Again, the same model systems, 4S genes on HeLa cell mitochondrial DNA and rRNA genes on ϕ80 transducing phages, were studied. The rather complex reaction scheme involves the following steps:

1. Conjugation of RNA to biotin. The RNA is oxidized at the 3′ end with periodate, and a Schiff base is formed by reaction with diaminopentane and stabilized by treatment with NaBH4. The distal amino group of the diaminopentane is acylated with NHS biotin (*N*-hydroxysuccinimide ester of biotin), and the resulting RNA-biotin conjugate purified by chromotography on avidin-sepharose.
2. Conjugation of ferritin to avidin. Ferritin is bromoacetylated by reaction with NHS bromoacetic acid in DMSO. Sulfhydryl groups are added to avidin by acylation with dithiodiacetic acid, followed by reduction with DTT. Covalent coupling of the modified ferritin to the modified avidin is achieved by mixing the above two.
3. Hybridization of biotin-RNA to DNA. The biotin-coupled RNA is hybridized to single-stranded HeLa mitochondrial DNA[143] or to ϕ80 heteroduplex molecules.[154] After hybridization, excess RNA-biotin had to be removed by passage over a Sepharose 2B column.
4. Labeling of the biotin-RNA:DNA hybrids with ferritin-avidin. Labeling of hybrids with ferritin-avidin is achieved by mixing equal volumes of hybrid and ferritin-avidin. This corresponds to a 5 to 10,000-fold excess of ferritin-avidin over the hybridized RNA-biotin, which is required for efficient labeling. Excess ferritin-avidin is removed by centrifugation through a solution of sodium iothalamate (5 acetamide-2,4,6 Triiodo-*N*-methyliso phthalamic acid).
5. Electron microscopy. Labeled molecules are mounted for electron microscopic observation by the formamide cytochrome spreading method. An example is shown in Figure 17.

The advantage of the RNA-biotin:avidin-ferritin tagging method over direct ferritin tagging is that the biotin molecule has a much smaller molecular weight (mol wt = 224) than ferritin (mol wt = 9×10^5) and should permit a much more efficient hybridization of the labeled RNA to the DNA. Even then the efficiency of the method is only 40 to 50%[143,154] Still, it has allowed more accurate mapping of 12 4S genes on the heavy strand as well as 7 4S genes on the light strand of mitochondrial DNA.[143]

Improved efficiency of labeling (up to 60%) was obtained by a modified method.[155] The modification consists in using cytochrome c for coupling biotin to the oxidized 3′ end of RNA, instead of the pentane diamine. The cytochrome c bridge is probably more extended and allows attachment of several biotins to one RNA molecule. Furthermore, it allows one to label tRNA:DNA hybrids with avidin attached to polymethacrylate spheres.

Avidin coupled polymethacrylate spheres had been used earlier for *in situ* hybridization experiments to label *Drosophila melanogaster* rRNA genes in chromosome preparations.[156] The biotin:avidin reaction has also been used for gene enrichment.[157] *Drosophila* histone mRNA was coupled via a cytochrome c bridge to biotin. R-loops were then formed with this modified mRNA and total nuclear DNA. Following the R-loop hybridization, avidin-spheres were added, and after the reaction the sphere-coupled R-loops were banded in a CsCl gradient. R-loop molecules formed with cloned *D. melanogaster* rRNA genes and biotin coupled rRNA were also examined in the

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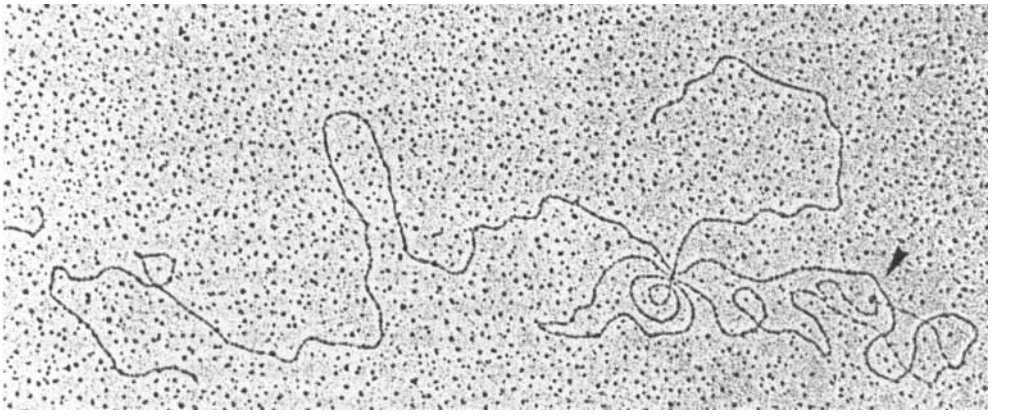

FIGURE 17. Labeling of tRNA genes with the ferritin-avidin:biotin tagging method. Biotinyl-tRNA^{Tyr} was hybridized to the rRNA gene on a ϕ80psuIII$^+$/ϕ80 heteroduplex and labeled with ferritin-avidin conjugate.[143,154] (Courtesy of T. Broker and L. Chow.,Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1979.)

electron microscope.[157] The results showed that although the yield of R-loop molecules is slightly decreased (from 81% to 67%), the biotin conjugated mRNA can efficiently form specific R-loops and is still able to interact with avidin-spheres.

The avidin-biotin reaction can also be applied for visualizing proteins bound to DNA. The small basic protein covalently attached to poliovirus double-stranded RNA was coupled to biotin and then labeled with avidin spheres.[158] Similarly, covalently bound terminal proteins of adenovirus-2 and phage ϕ29 have been labeled with ferritin-biotin:avidin complexes (Figure 18).[198]

C. Ferritin-Antibody Labeling

Ferritin-conjugated anitbodies have been used to demonstrate the binding of SV40 T antigen to the origin of replication of SV40 DNA.[159] Partially purified T antigen was incubated with the DNA and the complexes fixed with glutaraldehyde. They were first interacted with hamster anti-Ty-globulin. These labeled complexes were again fixed with glutaraldehyde and dialyzed into phosphate buffer. After spreading with 40% formamide in a cytochrome monolayer, the ferritin labeled T-antigen could be localized on the DNA molecules. For mapping of the binding site, the SV40 DNA (form I) with ferritin cores bound to T-antigen were sequentially reacted with *E.coli* DNA unwinding protein and restriction endonuclease *EcoRI* or *HpaII* to yield linear molecules with both a ferritin core and an unwinding protein melted region.[159]

D. DNP-Anti-DNP Labeling

An alternative method for labeling proteins attached to DNA by the use of antibodies was recently described by Wu and Davidson.[160] The method is based on the attachment of the dinitrophenyl (DNP) hapten to amino residues (mostly of lysine and perhaps other amino acids) of the protein by reaction with dinitrofluorobenzene (DNFB) followed by binding of rabbit anti-DNP Ig molecules to the DNP haptens. A second antibody label with goat antirabbit IgG can be used to increase the size of the complexes.

Dinitrophenylation reaction with proteins bound to nucleic acids has been studied under a variety of conditions in different buffers.[160] Satisfactory results were obtained under the following conditions: DNA, RNA, or protein were dialyzed against 0.125 *M* borate buffer (pH 9.0), 6.25 m*M* EDTA, and 10% v/v DMSO. DNFB stock solu-

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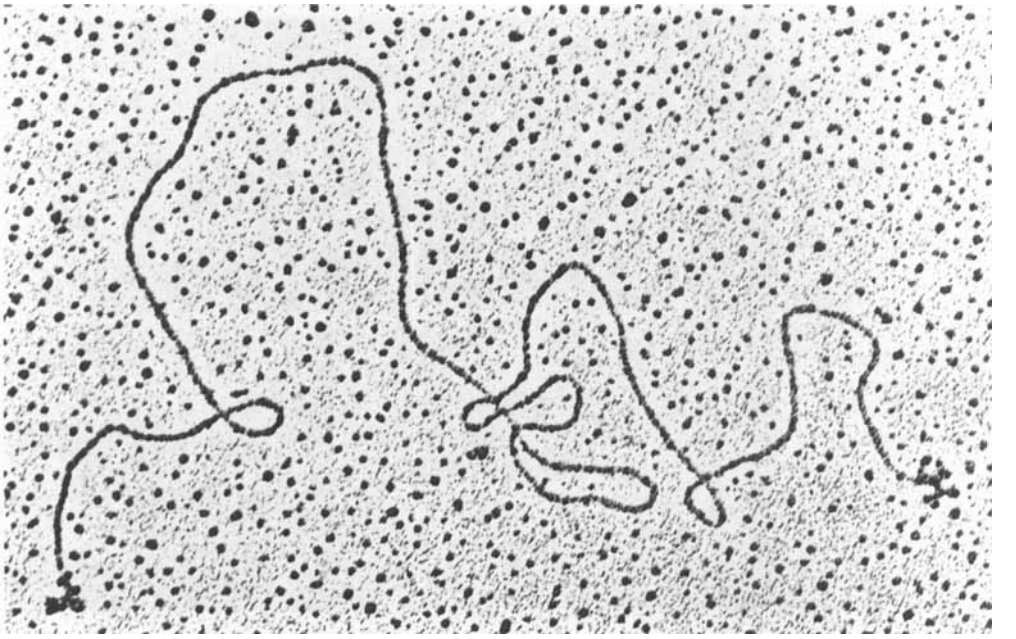

FIGURE 18. Ferritin-avidin:biotin labeling of the terminal proteins on *Bacillus subtilis* phage ϕ29 DNA. (Courtesy of T. Broker and L. Chow, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1979.)

tion (in ethanol) was added and the mixture incubated in the dark at 37°C. The extent of reaction can be controlled by varying the reaction time, e.g., from 6 to 30 DNP molecules were bound per ovalbumin in 10 to 60 min incubation. The excess DNFB was removed by ether extraction.

For observation of proteins that are not covalently bound to nucleic acids, the protein-DNA complexes were first fixed with 0.2% glutaraldehyde and all subsequent steps were carried out in the presence of 0.02% glutaraldehyde. For optimum results the entire process, including spreading, should then be completed within 12 hr.

The DNP modified protein-nucleic acid complexes were allowed to react with rabbit anti-DNP IgG by adding a large excess of antibody (200 μg anti-DNP per 0.5 to 2 μg of complex) and incubation at 37°C for 1 hr. Unbound protein was removed by gel filtration in Sepharose 2B or by centrifugation in CsCl. In some cases the labeled complexes were large enough to be clearly seen in the cytochrome spread samples. If not, they were amplified by a second labeling with goat-antirabbit IgG. In that case, anti-DNP labeled protein-DNA complexes were fixed with 0.02% gluteraldehyde, mixed with an equal volume of 10 mg/m*l* of the second antibody, incubated at 30°C for 1 hr, and passed again over a Sepharose 2B column.

The method has been applied to many systems, involving proteins covalently linked to DNA and RNA, as well as noncovalently bound proteins. The terminal protein of adehovirus-2 DNA was labeled with an efficiency of 78%.[160] It was possible to modify this protein with sufficient DNP groups so that several anti-DNP molecules bound at each end and produced a large enough aggregate to be seen. SV40-bound protein had to be amplified by second antibody labeling.[160] The small protein covalently attached to the 5′ end of poliovirus RNA, or to both ends of the double-stranded replicative form was visualized after a second reaction with a monovalent Fab fragment of goat-antirabbit IgG.[161] The use of monovalent Fab fragments for the second reaction prevents the formation of large aggregates of molecules that were pobably produced by

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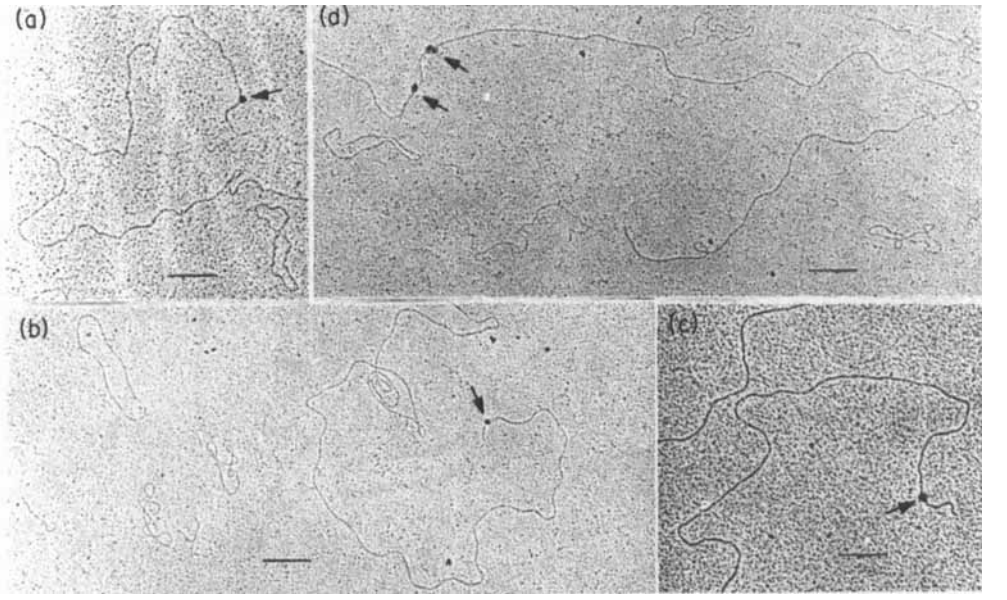

FIGURE 19. The subterminal protein on herpes simplex virus was visualized with the DNP tagging method.[160] The complex was treated with DNFB, rabbit anti-DNP IgG, and the Fab fragment of goat anti-rabbit IgG. (From Wu, M., Hyman, R. W., and Davidson, N., *Nucleic Acids Res.*, 6, 3427, 1979. With permission.)

interaction of free antibodies in solution with the two labeled DNA ends. The same method was used to identify the positions of a protein that is covalently bound near the end of herpes simplex virus DNA[162] (Figure 19). Mapping of these protein binding sites was done on partially denatured HSV and on fold-back molecules.

Mapping of noncovalently bound proteins requires cross-linking of the complex with glutaraldehyde and the presence of glutaraldehyde in all reactions to prevent displacement of cross-links with DNFB. Labeling of *E.coli* DNA polymerase I bound to 3′ ends of DNA molecules was achieved with an efficiency of ~55% with anti-DNP antibodies alone.[160] Similar efficiencies were obtained for visualization of *E.coli* RNA polymerase bound to T7 DNA and for proteins cross-linked by UV irradiation to avian sarcoma virus DNA.[160]

The DNP-anti-DNP labeling method seems to be simpler and of higher labeling efficiency than the previously described tagging methods. It allows handling of complexes by conventional cytochrome spreading methods. Even proteins as small as the poliovirus bound protein of 6000 mol wt can be sufficiently amplified with the double-labeling method. Such small proteins could never be detected with any of the protein-free spreading techniques.

IX. ACTIVE GENES

All the methods discussed so far give information about the structural organization of genetic material. Once the structure and the arrangement of specific genes has been established, one would also like to gain information about their functional organization. Many questions concerning gene function — particularly in view of the mosaic nature of many eukaryotic genes — remain to be answered, for example: definition of transcriptional units in mosaic genes; do eukaryotic genes have specific promoters like prokaryotic genes, or are specific transcription products created by well-defined

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splicing mechanisms? How does the processing of long precursor mRNAs proceed? How is transcription regulated? And what is the structure of actively transcribed genetic material? A number of recently developed electron microscope techniques try to approach some of these questions.

Several methods have been used for the mapping of promoter sites and analysis of transcription mainly on bacteriophage DNA: binding of RNA polymerase to DNA and visulization of the protein DNA complexes,[35,42,44,57,164] spreading of in vitro transcription complexes,[62,164,165] and mapping of transcription R-loops[166] or RNA:DNA hybrids made from in vitro transcripts. [167] It is hoped that some of these methods may also be adapted to the analysis of eukaryotic transcription systems, once the purified in vitro systems will be available. Another approach has been the direct visualization of active genes by spreading the in vitro transcribed genes with the elegant technique developed by Miller and Beatty.[52] Conventional R-loop mapping has allowed one to understand the complex processing pathways of adenovirus mRNAs.[124,126]

A. Visualization of In Vitro Transcription

Davis and Hyman[167] used two different methods for the direct observation of in vitro transcription of T7 DNA. When the transcription complexes DNA-RNA polymerase-RNA were mounted by the basic protein film method, the nascent transcripts were seen as collapsed RNA bushes on the duplex DNA. The number of RNA bushes on each template, the bush position relative to one end of the DNA, and the approximate RNA size (measured by bush size) were measured as a function of time of synthesis. In the second experiment, in vitro synthesized RNA was hybridized to separated strands of T7 DNA and the hybrid molecules spread either by the aqueous or the formamide method. These two experiments allowed accurate physical mapping of an initiation site for the *E.coli* RNA polymerase on T7 DNA, of the direction of transcription, and of termination sites in the presence of termination factor ρ. The smallest measurable bush was 400 nucleotides long, and the smallest detectable RNA:DNA hybrid was about 100.

With conventional spreading methods RNA molecules are not fully extended (because of extensive secondary structure formation) and it is not possible to accurately measure the length of individual RNA molecules in a transcription complex. Delius et al. [62,164] have described a technique for extending RNA chains using the bacteriophage T4 gene-32 protein.[168] This protein binds tightly and cooperatively to single-stranded DNA and to RNA and thereby removes the secondary structure.[62,86] The experiment was done as follows: DNA was transcribed in vitro with *E.coli* RNA polymerase, the transcription complexes were then diluted into potassium phosphate buffer containing the gene-32 protein and incubated at 37°C to let the protein bind to RNA. In order to stabilize the complexes, they were fixed with 0.1% glutaraldehyde before being spread from a 30% formamide solution onto 10% formamide hypophase. After fixation, the complexes can be purified on a Sepharose column to remove excess gene-32 protein without significant loss of transcripts. In the microscope the RNA chains appear as extended protein coated branches thicker in diameter than the duplex DNA (Figure 20). The smallest transcripts visible by this method are a few hundred nucleotides long. It is also possible to spread the 32 protein-coated transcription complexes by adsorbing them on mica by the divalent cation method;[61] this increases the resolution of the smallest RNA chains detectable.[63]

More elaborate than their preparation is the analysis of these complexes.[62,164] Plotting the lengths of all RNA molecules on individual transcription complexes against their distance from one end of the DNA molecule, one can extrapolate to the position of the promoter site (transcription initiation site). While RNA length measurements

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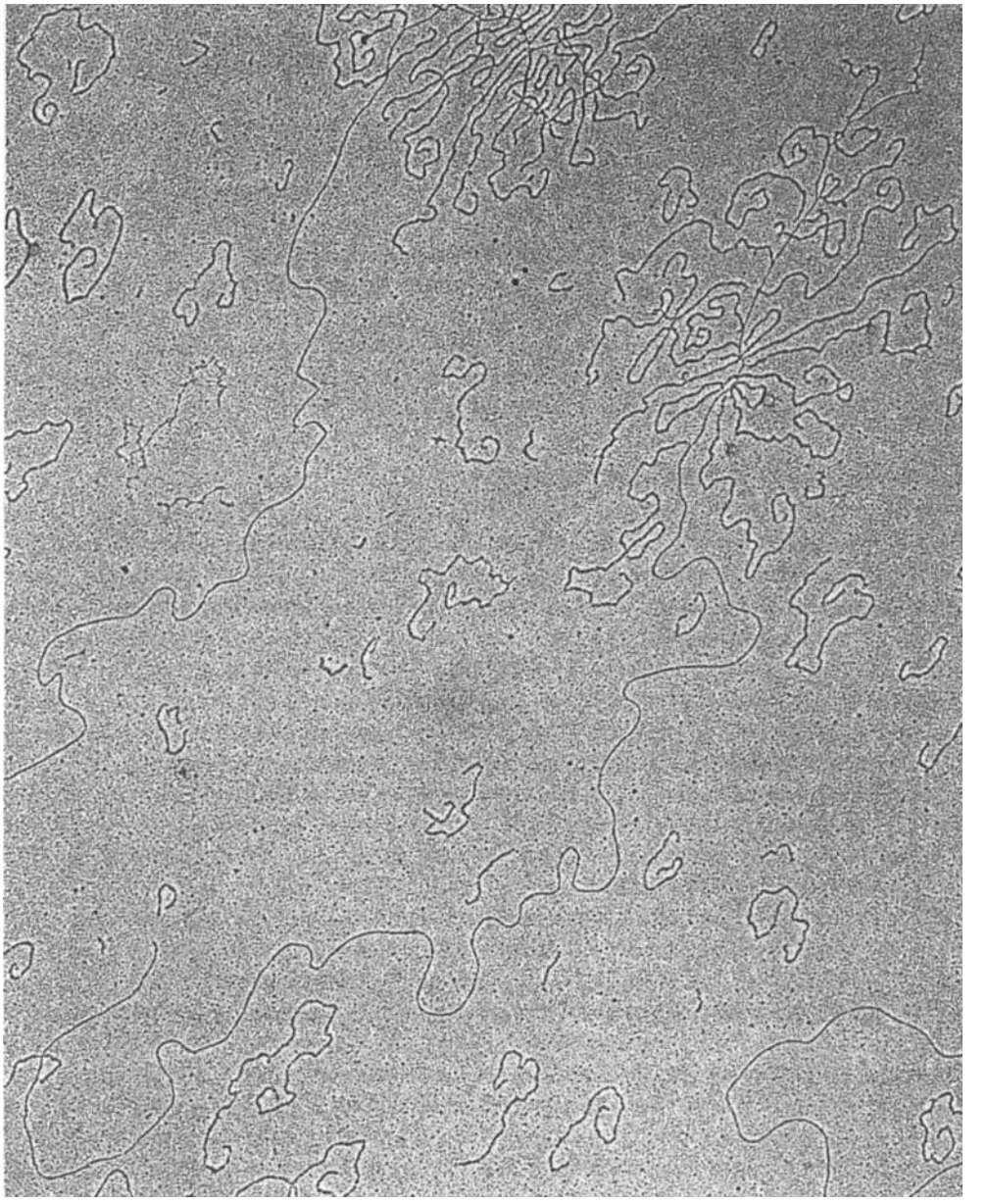

FIGURE 20. In vitro transcription complex of T7 DNA transcribed with *E. coli* RNA polymerase. The complexes were incubated with T7 gene-32 protein, fixed with gluteraldehyde, and spread with formamide-cytochrome, as described.[62] (Courtesy of H. Delius, European Molecular Biology Laboratory, Heidelberg, Germany, 1979.)

within individual transcription complexes were very consistent (error ± 4.8%), larger variations were obtained in the ratio of RNA length to transcribed DNA length when different molecules were compared, leading to an average error of ± 12.5%.[62]

The gene-32 protein method has been used to study transcription with *E.coli* RNA polymerase on phage T5 and T7 DNA,[62,164] on SV 40 DNA,[62] and on superhelical vs. linear λDNA.[165] It allows mapping of promoter sites and determination of the direction of RNA systhesis, of the elongation rate, and of the efficiency of transcription from

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different promoters. It was found that certain promoters are transcribed much more efficiently from superhelical λDNA than from a linear template[165] and that the *E.coli* enzyme can transcribe SV40 DNA into long RNA molecules by progressing several times aroung the circular template.[62] In addition to the availability of T4 gene-32 protein, other problems are encountered with this method. One is the fact that with increasing complexity of the transcription patterns, elaborate computation methods are needed to analyze the electron micrographs. A detailed description of such a computer analysis was recently published by Stüber, et al. [164] who have been able to map 40 promoter sites on T5 DNA. Another problem is that different promoters may be transcribed with widely different efficiency by *E.coli* RNA polymerase, leading to a large variation in the number of RNA chains per transcription unit. It may be difficult to determine the direction of transcription from regions which are rarely transcribed.

The transcription R-loop method recently described by Brack combines transcription with R-loop mapping techniques. [166] The method was developed on a model system of bacteriophage λDNA transcribed with *E.coli* RNA polymerase. DNA is transcribed in vitro and aliquots are removed from the reaction mixture at different time points. They are diluted directly into R-loop buffer containing 70% formamide and 0.56 *M* NaCl and incubated at 56°C. The transcription complexes are stable even under these high formamide and salt concentrations, and the RNA transcripts immediately hybridize to their template strand and form R-loops. At saturating ratios of enzyme to DNA, it is possible to obtain up to 96% R-loop molecules after a 10-min incubation of complexes in R-loop buffer. These transcription R-loop molecules can be further processed and mounted for electron microscopic observation like conventional R-loops. An example is shown in Figure 5. Measurement of the posititon and length of R-loops allows direct mapping of the transcription initiation sites (promoter sites) and of the direction and the rate of transcription. The level of transcription from different promoters indicates the affinity of the *E.coli* RNA polymerase for these promoters under the given experimental conditions. In simple systems, like the phage λ genome, relatively few measurements were needed to map the different promoters with an accuracy of 1 to 3%.[166] The smallest detectable R-loops were 70 to 100 nucleotides long.

For frequently transcribed genes, it is possible with the gene-32 protein method to read the direction of transcription from a single transcription complex. With our transcription R-loop method, however, one must compare a population of molecules with different size transcripts, either from one sample or from several samples taken at different times of synthesis. Measurement and analysis of R-loop molecules are straightforward and do not require sophisticated computation. Again, however, it may not always be possible to determine the direction of transcription; e.g., when short transcription units have strong termination sites resulting in similar size R-loops. As in other mapping experiments, there may be problems of orientation of linear molecules. They can be solved by comparing transcription patterns on different overlapping restriction fragments. Multiple initiation at one promoter may lead to complex double or triple loop structures that are difficult to interpret. Another problem may arise when transcripts originate from regions with different base composition. The conditions chosen for R-loop formation may be optimal for an A + T rich gene, in which case G + C rich transcripts may not completely hybridize and appear as RNA tails. Modification of the incubation conditions — e.g., by the two-step method or by gradual temperature shift through the Tss — should allow one to find conditions for optimal hybridization of different transcripts.

So far, the transcription R-loop method has been successfully used to map promoters on phage λDNA and different bacterial plasmids.[166] Promoters for phage P1 modification and restriction genes have been mapped.[199] Specific transcription initia-

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tion sites for the *E. coli* polymerase have been mapped at the beginning of cloned *Drosophila melanogaster* heat shock genes.[169] Other eukaryotic systems are presently being examined.[200] Since this method is simple and rapid and requires very little material (0.1 to 0.2 μg of DNA per experiment), it should be possible to use it as an assay to look for specific transcription in crude eukaryotic transcription systems and for purification of specific RNA polymerases.

B. Visualization of In Vivo Transcription

In 1969 Miller and Beatty published their spectacular micrographs of active nucleolar genes.[52] The method of direct visualization of in vivo transcribed ribosomal genes was then applied to different prokaryotic and eukaryotic systems.[52,170-172] A detailed description of the preparative techniques for isolation of nuclear contents and preparation of electron microscope samples is given in Reference 173. Other modifications of the technique, such as the microspreading technique,[173] have been described in experiments dealing with various cell types.[174-177] Further references can be found in these papers.

The "Miller technique" has also found wide applications in the field of chromatin structure and function. Volume 42 of the Cold Spring Harbor Symposium on Chromatin[178] contains a number of papers in which this technique was employed to study the structure of transcriptionally active chromatin.

Of particular interest is the application of the Miller technique to some experiments which Trendelenburg et al. have designed to analyze expression of cloned eukaryotic genes in a heterologous in vivo system.[179,180] Mertz and Gurdon had shown that purified xenogeneic DNA injected into amphibian oocytes is specifically transcribed.[181,182] When nuclear spreads were made from frog oocytes that had been injected with circular DNA containing amplified rRNA genes purified from insects, it was possible to recognize initial transcripts on the injected DNA and to analyze the specificity of the system[180] (e.g., Figure 21). It is important to inject circular DNA so that it can be determined whether the observed transcription complexes originate from injected or from endogenous DNA. It has also been shown that mitochrondrial DNA can be assembled into chromatin-like structures but is hardly transcribed when injected into oocyte nuclei.[183] If this system proves to be specific, it is a very promising approach to gain information about the transcriptional activity of cloned single-copy genes, the structure of the initial transcripts, and their processing.

X. PROTEIN-DNA INTERACTION

The importance of protein-nucleic acid interaction for the regulation of biological processes and the structural organization of genetic material has stimulated the development of many specialized electron microscopic methods for the visualization of DNA-protein complexes. Binding may be specific, as with complexes with enzymes and regulatory proteins controlling and affecting gene activity, or it may be nonspecific, as is the case with histones and other DNA binding proteins. As discussed in Section II of this review, the commonly used preparation method, spreading in a protein monolayer, does not allow visualization of small proteins bound to nucleic acids because the molecules are embedded in a coat of cytochrome c about 10 to 20 nm thick. Therefore, protein-free spreading methods like adsorption or BAC spreading methods have usually been used to prepare protein-nucleic acid complexes. Recently, development of the tagging or specific labeling methods described above have allowed the use of the conventional cytochrome spreading methods as well. In the following we shall discuss some protein-DNA interactions that have been studied with direct

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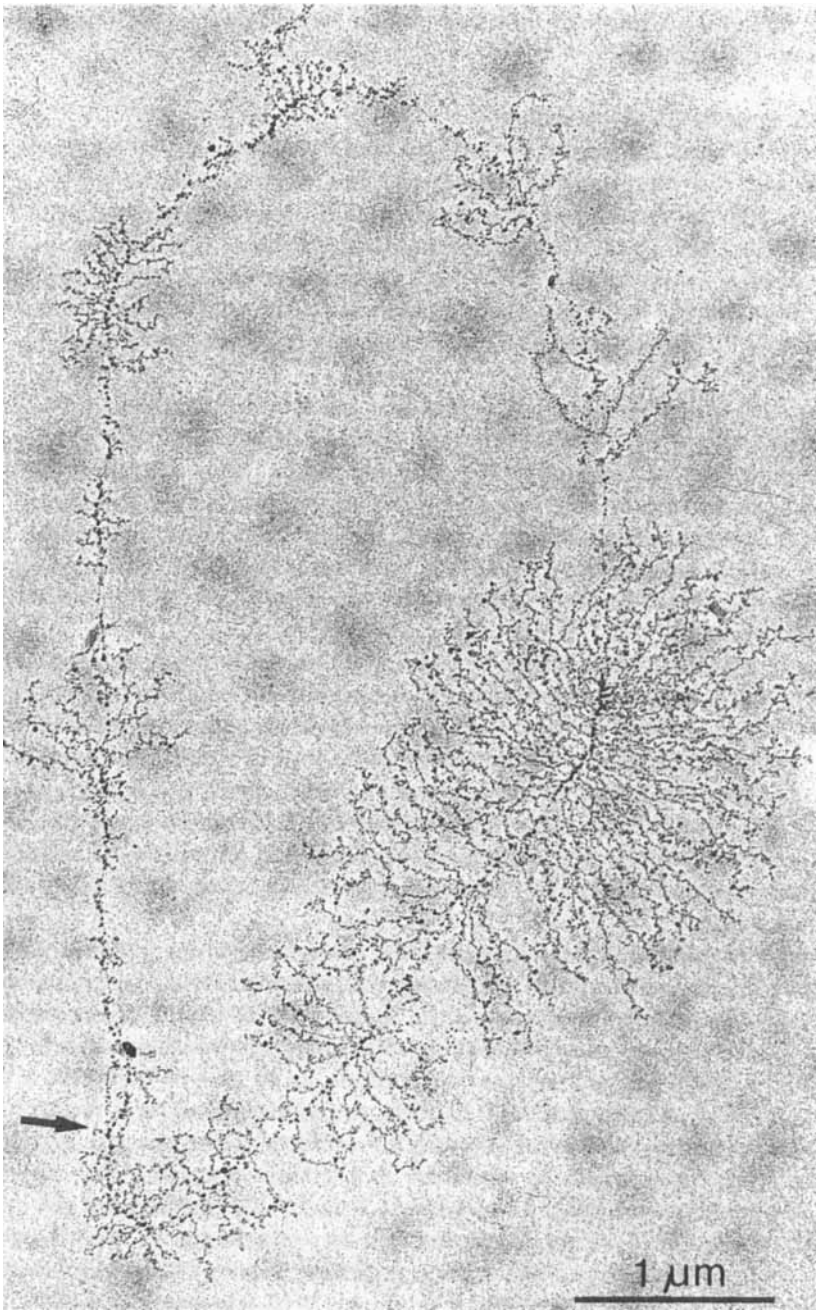

FIGURE 21. In vivo transcription complex. Spread preparation of a *Xenopus* oocyte nucleus after injection with purified rDNA. The circular molecule exhibits a dense array of RNP fibrils arranged in a regular length gradient. In addition, irregular clusters of lateral fibrils are seen in positions corresponding to spacer regions. (From Trendelenburg, M. F., Zentgraf, H., Franke, W. W., and Gurdon, J. B., *Proc. Natl. Acad. Sci. U.S.A.*, 75, 3791, 1978.)

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mounting methods. Additional information can be obtained in the recent review of Griffith and Christiansen.[37]

A. Specific Protein-DNA Interaction

Probably the most frequently studied protein-DNA complex that has been used as a model system for the development of many new techniques is the complex formed between the RNA polymerase of *E.coli* with specific promoter sequences on a variety of phage DNAs. Almost all of the techniques described in II.B have been used to map the early promoters on T7 DNA: the BAC spreading method,[57] the divalent cation[61] and ethidium bromide method,[57] adsorption to activated carbon films, and dark field examination.[19,42,44] Binding of the *E.coli* polymerase to λDNA has been studied by the BAC method.[35]

Compared to transcription mapping methods, the binding studies have the following disadvantage: polymerase binding sites can be mapped, but sites that actively initiate transcription cannot be discriminated from other binding sites. Certain promoter sites may initiate transcription very effectively but never form stable complexes; on the other hand, strong binding sites may not efficiently initiate transcription. The interaction of *E.coli* DNA polymerase with DNA has been examined by direct adsorption to activated carbon films,[184] and the Qβ replicase was mapped on Qβ RNA with the BAC spreading method.[36]

Brack and Pirrotta[43] studied the interaction of λ repressor with operator DNA. The repressor-DNA complexes were adsorbed onto pentylamine glow-discharged carbon films and first visualized in dark field after uranyl acetate staining for mapping of binding sites on total λDNA. Additional high resolution studies in bright field were done on complexes stained with uranyl formate. Weak staining with 0.5% uranyl formate on the hydrophobic carbon films resulted in positive staining of the DNA and negative staining of the protein molecules and permitted resolution of the subunits of the repressor tetramer (Figure 22). Several tetramers were observed to bind to the multiple operator sites.

Electron microscopy has helped to elucidate many steps in the complex pathway of the restriction endonuclease *EcoK* interacting with substrate DNA. Specific recognition sites for the enzyme have been mapped on λ and PM2 DNAs by direct visualization of recognition complexes adsorbed to positively charged carbon films after metal shadowing.[47] With highly purified enzyme preparations, up to 70 to 80% specific complexes could be observed. Addition of ATP to these recognition complexes results in a conformational change of the enzyme allowing it to proceed to the cleavage reaction. This conformational change could be visualized as a change in enzyme size: the diameter of the bound enzyme decreased from 1.6 to 1.2 nm.[185] The method did not allow one to determine whether this change was due to a loss of subunits or to a change in orientation or specific volume of the protein molecule. Figure 23 shows these different complexes: (a) recognition complexes of *EcoK* on λDNA fragments, and (b) the smaller cleavage complex (or filter binding complex) after reaction wih ATP.[185] Intermediates in the cleavage reaction, highly supercoiled DNA molecules, have recently been visualized in the electron microscope.[201]

Specific proteins bound to the origin of replication on SV40 DNA have been visualized directly[51] or with ferritin-antibody labeling,[159] and proteins covalently bound at the end of adenovirus DNA,[160] poliovirus RNA,[161] and near the ends of herpes simplex virus DNA[162] have been localized with various tagging methods (Figures 18,19). Binding sites for two specific nonhistone DNA binding proteins purified from *D. melanogaster* have been mapped on cloned *Drosophila* DNA fragments[186,187] (Figure 24).

What is the minimum size of a protein that can be detected in a protein-DNA com-

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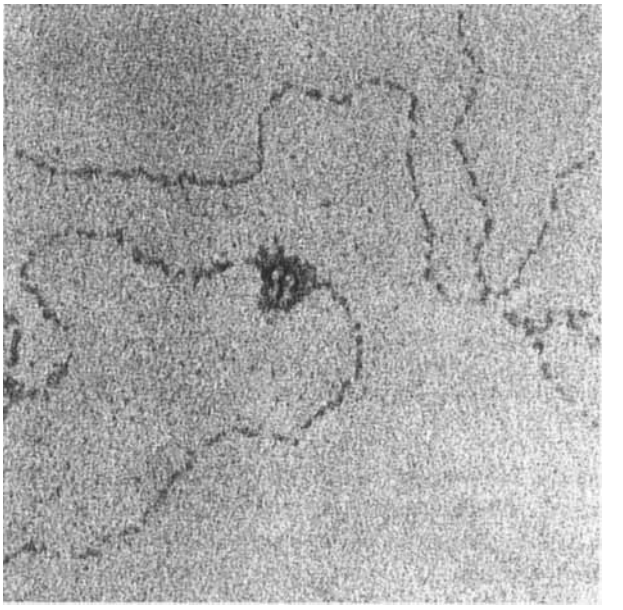

FIGURE 22. Specific complex between λ repressor and λ operator DNA. The complex was prepared by adsorption to pentylamine glow-discharged carbon film and stained with 0.5% aqueous uranyl formate.[43] The DNA is positively stained and the repressor molecule appears negatively stained; the four subunits of the repressor tetramer can be clearly distinguished.

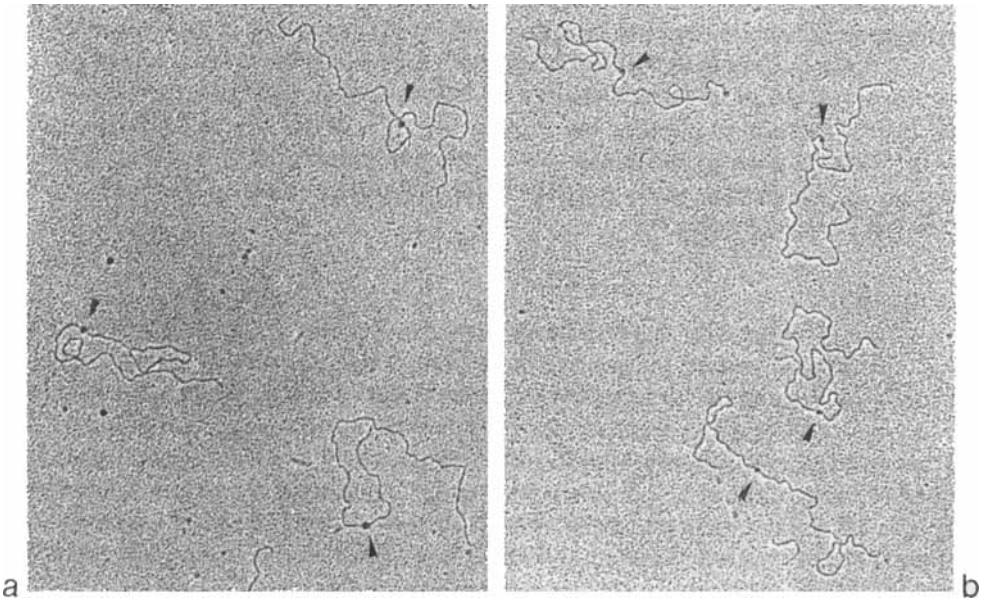

FIGURE 23. (a) Specific binding of the restriction endonuclease *EcoK* to the recognition site on λ restriction fragments *EcoRI* f6. (b) ATP induces a conformational change in the enzyme that can be visualized as a decrease in the size of the enzyme (→).[185]

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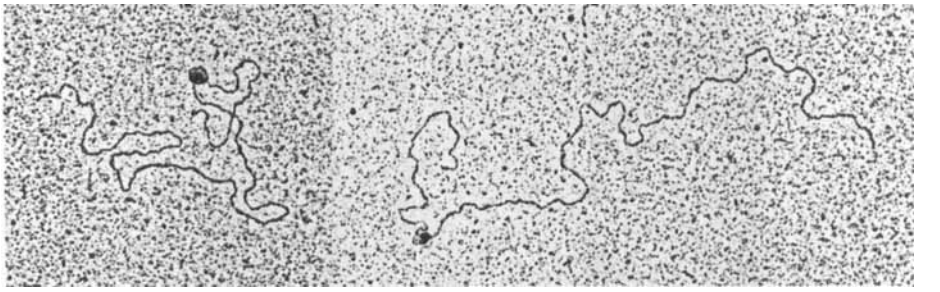

FIGURE 24. Specific binding of a protein purified from *Drosophila melanogaster* (R. Jack, unpublished results) to a cloned *Drosophila* DNA fragment (56H8[193]) containing the major heat shock gene. The specific complexes were adsorbed to pentylamine glow-discharged carbon films and rotary shadowed with platinum.[187]

plex by direct electron microscope mounting procedures? This depends not only on the molecular weight, but on many other properties of the proteins: partial specific volume and shape of the specific protein are some parameters. Depending on the preparation method used, the protein may be more or less deformed and flattened on the grid during adsorption, staining, and drying processes. As an example, the λ repressor tetramer with a molecular weight of ~120,000 daltons does not appear much smaller than RNA polymerase (molecular weight ~450,000 daltons) when complexes are adsorbed on pentylamine charged carbon films and stained with uranyl formate.[43] The RNA polymerase subunits are probably much more closely packed and cannot be easily distinguished, whereas repressor subunits are loosely associated and therefore more flattened on the grid.

Discrimination between specific and nonspecific binding of a protein to DNA can sometimes be achieved with the addition of polyanionic competitors like heparin.[185] The presence of heparin does not interfere with the adsorption of complexes to carbon grids, but it may inhibit cytochrome monolayer formation in the droplet spreading.

An important point in the preparation of DNA-protein complexes for electron microscopy, the fixation of samples, has been discussed in detail.[14] In general, the protein is cross-linked to DNA by incubation with 0.1% glutaraldehyde. Once fixed, the complexes are stable enough to be purified from unbound protein or contaminants by Sepharose chromatography.[45,62]

B. Chromatin

Discussion of chromatin structure goes beyond the scope of this review. Nevertheless, it must be emphasized that electron microscopy has contributed to the discovery of the nucleosome and the understanding of chromatin structure. The beaded structure of chromatin, composed of regularly spaced ν-bodies (nucleosomes) on DNA was discovered on negatively stained preparations of chromatin.[188] The structure of chromatin depleted of histone H1, of active chromatin, and of replicating chromatin and the role of histone H1 in the condensation of beaded chromatin into higher order structures, and many other features have been studied with various electron microscope methods. Volume 42 of the *Cold Spring Harbor Symposium on Chromatin* should be consulted for detailed information on this subject.[178] Figure 25 shows one example of chromatin prepared with the BAC spreading method.[60]

C. Other DNA Binding Proteins

A number of proteins have been isolated that bind specifically to single-stranded

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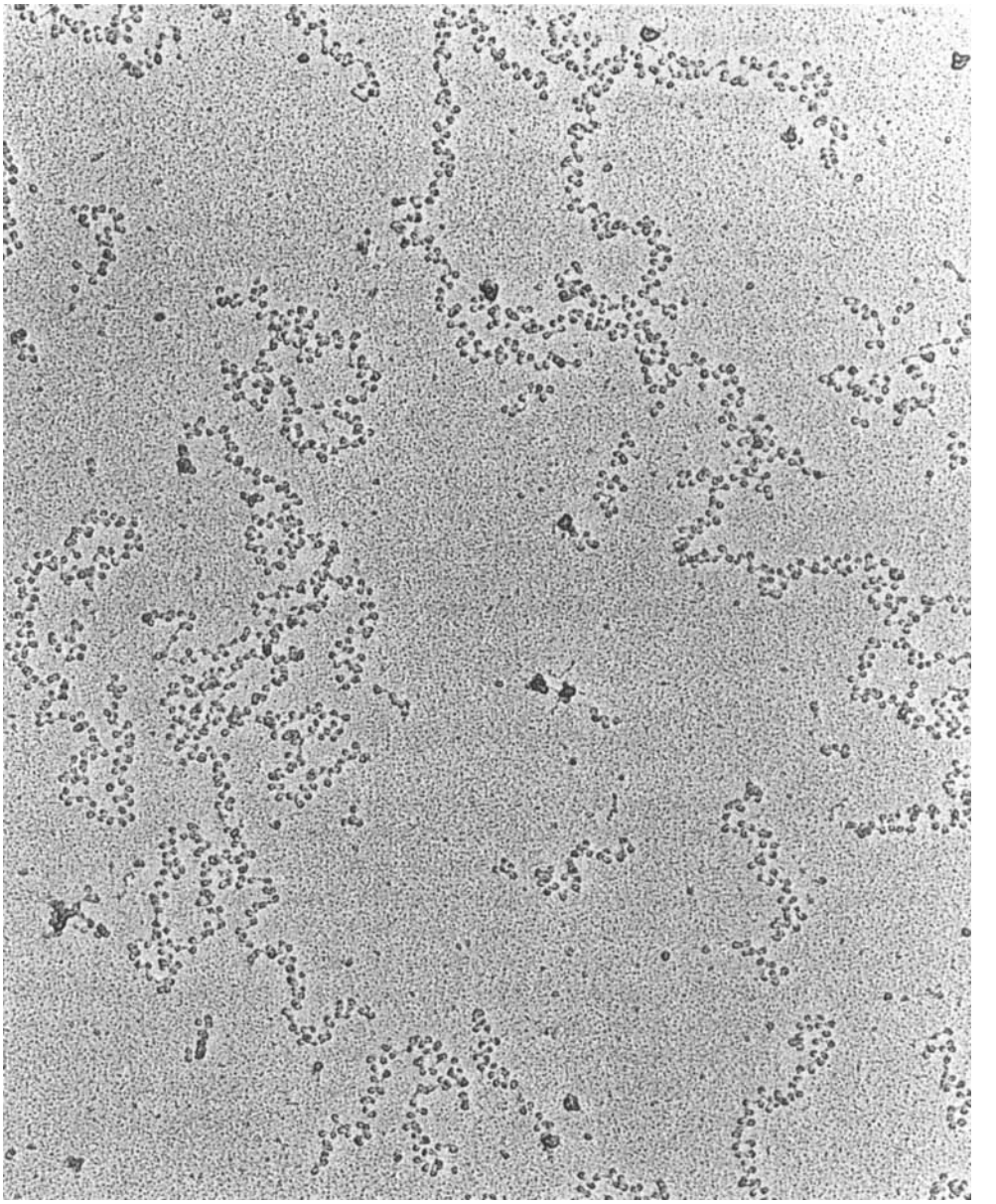

FIGURE 25. Rat liver chromatin was fixed with 0.1% glutaraldehyde (in 10m*M* NaCl, 0.2m*M* EDTA, and 5m*M* Triethanolaminchlorid, pH 7).[60] The chromatin was spread with 2×10^{-4}% BAC, picked up on freshly prepared carbon films, and rotary-shadowed with platinum. (Courtesy of T. Koller., Institut für Zellbiologie, ETH, Zurich, Switzerland, 1979.)

DNA and some of their properties have been characterized by electron microscopy. Delius et al.[86] have visualized the cooperative binding of the phage T4 gene-32 protein to single-stranded DNA. Its high affinity to single-stranded DNA and RNA has been used to denature DNA and extend RNA molecules.[45,62,88,165] Similar properties could be observed with the *E.coli* unwinding protein. [87] The gene-5 protein of filamentous bacteriophages (fd, M13) binds to single-stranded DNA in a different way: it coalesces two protein-covered DNA strands into helical, rod-shaped structures, which resemble the structure of mature phages.[189]

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Marcoli et al. have studied the interaction of phage PM2 protein IV with PM2 DNA.[190] This protein binds cooperatively to single-stranded DNA and to double-stranded supercoiled or relaxed DNA. The *E.coli* DNA binding protein HU was shown to condense DNA into chromatin-like beaded structures.[46]

XI. CONCLUSIONS

As this review has shown, electron microscopic analysis of DNA molecules has developed quickly since the seminal observations made by Kleinschmidt 20 years ago. Several crucial methods, mostly based on nucleic acid hybridization, permit both qualitative and quantitative analysis of gene organization. Combination of these methods allows almost unlimited application to various problems. Limitations imposed by the conventional preparation methods can be partially overcome with specific labeling techniques. The advantages of electron microscopic methods are as follows. Very little material is used (of the order of nanograms) and the sample preparation is relatively fast, allowing a qualitative characterization within a few hours. Extensive quantitative analysis yields invaluable information complementing evidence obtained by biochemical and genetic methods. On the other hand, it provides essential primary information, e.g., in the genome organization, in the detection of important DNA regions, in the characterization of protein-DNA complexes, and in the analysis of specific transcription.

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REFERENCES

1. **Ferguson, J. and Davis, R. W.**, Quantitative electron microscopy of nucleic acids, in *Advanced Techniques in Biological Electron Microscopy II*, Koehler, J. K., Ed., Springer-Verlag, Berlin-Heidelberg, 1978, 123.
2. **Evenson, D. P.**, Electron microscopy of viral nucleic acids, *Methods Virol.*, 6, 219, 1977.
3. **Meyer, J.**, Electron microscopy of viral RNA, in *Current Topics in Microbiology and Immunology*, 1981, in press.
4. **Kleinschmidt, A. K. and Zahn, R. K.** Ueber Deoxyribonucleinsäure-Molekeln in Protein-Mischfilmen, *Z. Naturforsch.*, 14b, 770, 1959.
5. **Vollenweider, H. J., Sogo, J. M., and Koller, T.**, A routine method for protein-free spreading of double- and single- stranded nucleic acid molecules, *Proc. Natl. Acad. Sci. U.S.A.*, 72, 83, 1975.

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6. **Thomas, J. O., Kolb, A., and Szer, W.,** Structure of single-stranded nucleic acids in the presence of ribosomal protein S1, *J. Mol. Biol.*, 123, 163, 1978.
7. **Thomas J. O., Sternberg, N., and Weisberg, R.,** Altered arrangement of the DNA in injection-defective lambda bacteriophage, *J. Mol. Biol.*, 123, 149, 1978.
8. **Zollinger, M., Guertin, M., and Mamet-Bratley, M. D.,** A new electron microscope method for studying protein-nucleic acid interactions, *Anal. Biochem.*, 82, 196, 1977.
9. **Davis, R. W., Simon, M., and Davidson, N.,** Electron microscope heteroduplex methods for mapping regions of base sequence homology in nucleic acids, *Methods Enzymol.*, 21, 413, 1971.
10. **Thomas J. O.,** Electron microscopy of DNA, in *Principles and Techniques of Electron Microscopy*, Vol. 9, Hayat, M. A., Ed., Van Nostrand Reinhold, New York, 1978, 64.
11. **Kleinschmidt, A. K.,** Monolayer technique in electron microscopy of nucleic acid molecules, *Methods Enzymol.*, 12B, 361, 1968.
12. **Inman, R. B.,** Some factors affecting electron microscopic length of DNA, *J. Mol. Biol.*, 25, 209, 1967.
13. **Bujard, H.,** Electron microscopy of single-stranded DNA, *J. Mol. Biol.*, 49, 93, 1970.
14. **Inman, R. B. and Schnös, M.,** Partial denaturation of thymine- and 5-Bromouracil-containing λ DNA in alkali, *J. Mol. Biol.*, 49, 93, 1970.
15. **Davis, R. W. and Davidson, N.,** Electron microscopic visualization of deletion mutations, *Proc. Natl. Acad. Sci. U.S.A.*, 60, 243, 1968.
16. **Egel-Mitani, M. and Egel, R.,** A rapid visualisation of Kleinschmidt-type DNA preparations by phosphotungstic acid, *Z. Naturforsch.*, 27b, 480, 1972.
17. **Delain, E. and Brack, C.,** Visualization des molécules d'acides nucléiques. II. Microversion de la technique de diffusion, *J. Microsc. (Paris)*, 21, 217, 1974.
18. **Kleinschmidt, A. K.,** Electron microscopic studies of macromolecules without appositional contrast, *Philos. Trans. R. Soc. London Ser. B.*, 261, 143, 1971.
19. **Dubochet, J., Ducommun, M., Zollinger, M., and Kellenberger, E.,** A new preparation method for dark-field electron microscopy of biomacromolecules, *J. Ultrastruct. Res.*, 35, 147, 1971.
20. **Brack, C., Eberle, H., Bickle, T. A., and Yuan, R.,** A map of the sites of bacteriophage PM2 DNA for the restriction endonuclease HindIII and HpaII, *J. Mol. Biol.*, 104, 305, 1976.
21. **Barker, D. C.,** Evaluation of the dark field method for large associations of spread DNA, in *10th Int. Congr. Electron Microscopy*, Vol. 2, Toronto, 1978, 190.
22. **Westmoreland, B. C., Szybalski, W., and Ris, H.,** Mapping of deletions and substitutions in heteroduplex DNA molecules of bacteriophage lambda by electron microscopy, *Science*, 163, 1343, 1969.
23. **Philippsen, P., Thomas, M., Kramer, R. A., and Davis, R.,** Unique arrangement of coding sequences for 5s, 5.8s, 18s, and 25s rRNA in *Saccharomyces cerevisiae* as determined by R-loop and hybridization analysis, *J. Mol. Biol.*, 123, 387, 1978.
24. **Davis, R. W. and Hyman, R. W.,** A study in evolution: the DNA base sequence homology between coliphages T7 and T3, *J. Mol. Biol.*, 62, 287, 1971.
25. **Brack, C., Hirama, M., Lenhard-Schuller, R., and Tonegawa, S.,** A complete immunoglobulin gene is created by somatic recombination, *Cell*, 15, 1, 1978.
26. **McConaughy B. L., Laird, C. D., and McCarthy, B. J.,** Nucleic acid reassociation in formamide, *Biochemistry*, 8, 3289, 1969.
27. **Lang, D., Kleinschmidt, A. K., and Zahn, R. K.,** Konfiguration und Längenverteilung von DNA-Mölekulen in Lösung, *Biochim. Biophys. Acta*, 88, 142, 1964.
28. **Lang, D. and Mitani, M.,** Simplified quantitative electron microscopy of biopolymers, *Biopolymers*, 9, 373, 1970.
29. **Mazaitis, A. J., Palchaudhuri, S., Glansdorff, N., and Maas, W. K.,** Isolation and characterization of λ dargECBH transducing phages and heteroduplex analysis of the argECBH cluster, *Mol. Gen. Genet.*, 143, 185, 1976.
30. **Brack, C., Delain, E., Riou, G., and Festy, B.,** Molecular organization of the kinetoplast DNA of *Trypanosoma cruzi* treated with Berenil, a DNA interacting drug, *J. Ultrastruct. Res.*, 39, 568, 1972.
31. **Borst, P. and Hoeijmakers, J. H. J.,** Review: kinetoplast DNA, *Plasmid*, 2, 20, 1971.
32. **Portmann, R., Schaffner, W., and Birnstiel, M.,** Partial denaturation mapping of cloned histone DNA from sea urchin *Psammechinus miliaris*, *Nature (London)*, 264, 31, 1976.
33. **Portman, R.,** personal communication.
34. **Sogo, J. M., Rodeño, P., Koller, T., Viñuela, E., and Salas, M.,** Comparison of the A-T rich region and the bacillus subtilis RNA polymerase binding sites in phage ϕ29 DNA, *Nucleic Acid Res.*, 7, 107, 1979.
35. **Vollenweider, H. J. and Szybalski, W.,** Electron microscopic mapping of RNA polymerase binding to coliphage lambda DNA, *J. Mol. Biol.*, 123, 485, 1978.
36. **Vollenweider, H. J., Koller, T., Weber, H., and Weissmann, C.,** Physical mapping of Qβ replicase binding sites on Qβ RNA, *J. Mol. Biol.*, 101, 367, 1976.

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RIGHTSLINK

37. **Griffith, J. D. and Christiansen, G.**, Electron microscope visualization of chromatin and other DNA-protein complexes, *Annu. Rev. Biophys. Bioeng.*, 7, 19, 1978.
38. **Coetzee, W. F. and Pretorius, G. H. J.**, Factors which influence the electron microscopic appearance of DNA when benzyldimethylammonium chloride is used, *J. Ultrastruct. Res.*, 67, 33, 1979.
39. **Thach, S. S. and Thach, R. E.**, Mechanism of viral replication. I. Structure of replication complexes of R17 bacteriophage, *J. Mol. Biol.*, 81, 367, 1973.
40. **Koller, T., Harford, A. G., Lee, Y. K., and Beer, M.**, New methods for the preparation of nucleic acid molecules for electron microscopy, *Micron*, 1, 110, 1969.
41. **Whiting, R. F. and Ottensmeyer, F. P.**, Heavy atoms in model compounds and nucleic acids imaged by dark field transmission electron microscopy, *J. Mol. Biol.*, 67, 173, 1972.
42. **Bordier, C. and Dubochet, J.**, Electron microscopic localization of the binding sites of *Escherichia coli* RNA polymerase in the early promoter region of T7 DNA, *Eur. J. Biochem.*, 44, 617, 1974.
43. **Brack, C. and Pirrotta, V.**, Electron microscopic study of the repressor of bacteriophage λ and its interaction with operator DNA, *J. Mol. Biol.*, 96, 139, 1975.
44. **Brack, C. and Delain, E., Electron-microscopic mapping of AT-rich regions and of** *E. coli* RNA polymerase-binding sites on the circular kinetoplast DNA of *Trypanosoma cruzi*, *J. Cell Sci.*, 17, 287, 1975.
45. **Brack C., Bickle, T., and Yuan, R.**, The relation of single-stranded regions in bacteriophage PM2 supercoiled DNA to the early melting sequences, *J. Mol. Biol.*, 96, 693, 1975.
46. **Rouviére-Yaniv, J. and Yaniv, M.**, *E. coli* DNA binding protein HU forms nucleosome-like structure with circular double-stranded DNA, *Cell*, 17, 265, 1979.
47. **Brack, C., Eberle, H., Bickle, T. A., and Yuan, R.**, Mapping of recognition sites for the restriction endonuclease from *Escherichia coli* K12 on bacteriophage PM2 DNA, *J. Mol. Biol.*, 108, 583, 1976.
48. **Cremisi, C., Pignati, P. F., Croissant, O., and Yaniv, M.**, Chromatin-like structures in polyoma virus and simian virus 40 lytic cycle, *J. Virol.*, 17, 204, 1976.
49. **Griffith, J. D.**, Electron microscopic visualization of DNA in association with cellular components, in *Methods in Cell Biology*, Vol. 7, Prescott, P. M., Ed., Academic Press, New York, 1972, 129.
50. **Griffith, J. D.**, Chromatin structure: deduced from a minichromosome, *Science*, 187, 1202, 1975.
51. **Griffith, J., Dieckmann, M., and Berg, P.**, Electron microscope localization of a protein bound near the origin of simian virus 40 DNA replication, *J. Virol.*, 15, 167, 1975.
52. **Miller, O. L. and Beatty, B. R.**, Visualization of nucleolar genes, *Science*, 164, 955, 1969.
53. **Harford, A. G. and Beer, M.**, Electron-microscopic localization of the binding of *E. coli* RNA polymerase to T7 DNA in vitro, *J. Mol. Biol.*, 69, 179, 1972.
54. **Abermann, R. and Salpeter, M. M.**, Visualization of deoxyribonucleic acid molecules by protein film adsorption and tantalum-tungsten shadowing, *J. Histochem. Cytochem.*, 22, 845, 1974.
55. **Williams, R. C.**, Use of polylysine for adsorption of nucleic acids and enzymes to electron microscope specimen films *Proc. Natl. Acad. Sci. U.S.A.*, 74, 2311, 1977.
56. **Gordon, C. N.**, On the molecular length of the replicative form DNA of bacteriophage ϕX174, *J. Mol. Biol.*, 78, 601, 1973.
57. **Koller, T., Sogo, J. M., and Bujard, H.**, An electron microscopic method for studying nucleic acid-protein complexes. Visualization of RNA polymerase bound to the DNA of bacteriophage T7 and T3, *Biopolymers*, 13, 995, 1974.
58. **Wu, M. and Davidson, N.**, Use of gene 32 protein staining of single-strand poly-nucleotides for gene mapping by electron microscopy: application to the ϕ80d$_3$ ilvsu'7 system, *Proc. Natl. Acad. Sci. U.S.A.*, 72, 4506, 1975.
59. **Thoma, F. and Koller, T.**, Influence of histone H1 on chromatin structure, *Cell*, 12, 101, 1977.
60. **Thoma, F., Koller, T., and Klug, A.**, Involvement of histone H1 in the organization of the nucleosome and of the salt-dependent superstructures of chromatin, *J. Cell Biol.*, 83, 403, 1979.
61. **Portmann, R. and Koller, T.**, The divalent cation method for protein-free spreading of nucleic acid molecules, in *6th European Congr. Electron Microscopy*, Ben-Shaul, Y., Ed., Tal International publishing, Jerusalem, Israel, 1976, 546.
62. **Delius, H., Westphal, H., and Axelrod, N.**, Length measurements of RNA synthesized in vitro by *Escherichia coli* RNA polymerase, *J. Mol. Biol.*, 74, 677, 1973.
63. **Delius, H.**, personal communication.
64. **Robberson, D., Aloni, Y., Attardi, C., and Davidson, N.**, Expression of the mitochrondrial genome in HeLa cells. VI. Size determination of mitochrondrial ribosomal RNA by electron microscopy, *J. Mol. Biol.*, 60, 474, 1971.
65. **Maniatis, T., Jeffrey, A., and van de Sande, H.**, Chain length determination of small double- and single-stranded DNA molecules by polyacrylamide gel electrophoresis, *Biochemistry*, 14, 3787, 1975.
66. **Baumeister, W. and Hahn, M.**, Specimen supports, in *Principles and Techniques of Electron Microscopy*, Vol. 8., Hayat, M. A., Ed., Van Nostrand Reinhold, New York, 1978, 1.

Critical Reviews in Biochemistry and Molecular Biology Downloaded from informahealthcare.com by 89.163.34.136 on 01/06/12
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67. **Brack, C.,** Use of uranyl formate staining for the electron microscopic visualization of DNA-protein complexes, *Experientia,* 29, 768, 1973.
68. **Fiers, W., Contreras, R., Haegemann, G., Rogiers, R., Van de Voorde, A., Van Heuverswyn, H., Van Herreweghe, J., Volckaert, G., and Ysebaert, M.,** Complete nucleotide sequence of SV40 DNA, *Nature (London),* 273, 113, 1978.
69. **Reddy, V. B., Thimmappaya, B., Dhar, R., Subramanian, K. N., Zain, B. S., Pan, J., Ghosh, P. K., Celma, M. L., and Weissmann, S. M.,** The genome of simian virus 40, *Science,* 200, 494, 1978.
70. **Sutcliffe, G.,** Nucleotide sequence of pBR322, *Cold Spring Harbor Symp. Quant. Biol.,* 43, 77, 1980.
71. **Sanger, F., Coulson, A. R., Friedmann, T., Air, G. M., Barrell, B. G., Brown, N. L., Fiddes, J. C., Hutchison, C. A., Slocombe, P. M., and Smith, M.,** The nucleotide sequence of bacteriophage ϕX174, *J. Mol. Biol.,* 125, 225, 1978.
72. **Beck, E., Sommer, R., Auerswald, E. A., Kurz, C., Zink, B., Osterburg, G., Schaller, H., Sugimoto, K., Sugisaki, H., Okamoto, T., Takanami, M.,** Nucleotide sequence of bacteriophage fd DNA, *Nucleic Acid Res.,* 5, 4495, 1978.
73. **Stüber, D. and Bujard, H.,** Electron microscopy of DNA: determination of absolute molecular weights and linear density, *Mol. Gen. Genet.,* 154, 299, 1977.
74. **Sharp, P. A., Hsu, M., Ohtsubo, E., and Davidson, N.,** Electron microscope heteroduplex studies of sequence relations among plasmids of *Escherichia coli.* I. Structure of F-prime factors, *J. Mol. Biol.,* 71, 471, 1972.
75. **Raleigh, E. A. and Davis, R. W.,** Determination of DNA concentration by electron microscopy, *Anal. Biochem.,* 72, 460, 1976.
76. **Inman, R. B.,** A denaturation map of the λ phage DNA molecule determined by electron microscopy, *J. Mol. Biol.,* 18, 464, 1966.
77. **Inman, R. B.,** Denaturation maps of the left and right sides of the lambda DNA molecule determined by electron microscopy, *J. Mol. Biol.,* 28, 103, 1967.
78. **Gòmez, B. and Lang, D.,** Denaturation map of bacteriophage T7 DNA and direction of DNA transcription, *J. Mol. Biol.,* 70, 239, 1972.
79. **Yoshiike, K., Furuno, A., and Suzuki, K.,** Denaturation maps of complete and defective simian virus 40 DNA molecules, *J. Mol. Biol.,* 70, 415, 1972.
80. **Schnös, M and Inman, R. B.,** Position of branch points in replicating λ DNA, *J. Mol. Biol.,* 51, 61, 1970.
81. **Inman, R. B. and Schnös, M.,** Structure of branch points in replicating DNA: presence of single-stranded connections in λ DNA branch points, *J. Mol. Biol.,* 56, 319, 1971.
82. **Chattoraj, D. K. and Inman, R. B.,** Position of two deletion mutations on the physical map of bacteriophage P2, *J. Mol. Biol.,* 66, 423, 1972.
83. **Wolfson, J., Dressler, D., and Magazin, M.,** Bacteriophage T7 DNA replication: a linear replicating intermediate, *Proc. Natl. Acad. Sci. U.S.A.,* 69, 499, 1971.
84. **Delius, H. and Clements, J. B.,** A partial denaturation map of Herpes Simplex Virus Type 1 DNA: evidence for inversions of the unique DNA regions, *J. Gen. Virol.,* 33, 125, 1976.
85. **Melchior, W. B., Jr. and Von Hippel, P. H.,** Alteration of the relative stability of dA.dT and dG.dC base pairs in DNA, *Proc. Natl. Acad. Sci. U.S.A.,* 70, 298, 1973.
86. **Delius, H., Mantell, N. J., and Alberts, B.,** Characterization by electron microscopy of the complex formed between T4 bacteriophage gene 32-protein and DNA, *J. Mol. Biol.,* 67, 341, 1972.
87. **Sigal, N., Delius, H., Kornberg, T., Gefter, M. L., and Alberts, B.,** A DNA-unwinding protein isolated from *Escherichia coli*: its interaction with DNA and with RNA polymerase, *Proc. Natl. Acad. Sci. U.S.A.,* 69, 3537, 1972.
88. **Hosoda, J., Takacs, B., and Brack, C.,** Denaturation of T4 DNA by an in vitro processed gene 32-protein, *FEBS Lett.,* 47, 338, 1974.
89. **Johnson, D.,** A new method of DNA denaturation mapping, *Nucleic Acid Res.,* 2, 2049, 1975.
90. **Schnös, M. and Inman, R. B.,** Starting point and direction of replication in P2 DNA, *J. Mol. Biol.,* 55, 31, 1971.
91. **Meyer, J., Iida, S., and Arber, W.,** Does the insertion element Is transpose preferentially into A-T-rich DNA segments? *Molec. Gen. Genet.,* 178, 471, 1980.
92. **Wellauer, P. K., Reeder, R. H., Dawid, I. B., and Brown, D. D.,** The arrangement of length heterogeneity in repeating units of amplified and chromosomal DNA from Xenopus laevis, *J. Mol. Biol.,* 105, 487, 1976.
93. **Mertz, J. E., Carbon, J., Herzberg, M., Davis, R. W., and Berg, P.,** Isolation and characterization of individual clones of simian virus 40 mutants containing deletions and insertions in their DNA, *Cold Spring Harbor Symp. Quant. Biol.,* 39, 69, 1974.
94. **Lee, C. S., Davis, R. W., and Davidson, N.,** A physical study by electron microscopy of the terminally repetitious, circularly permuted DNA from the coliphage particles of *Escherichia coli* 15, *J. Mol. Biol.,* 48, 1, 1970.

Critical Reviews in Biochemistry and Molecular Biology Downloaded from informahealthcare.com by 89.163.34.136 on 01/06/12
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95. **Hozumi, N., Brack, C., Pirrotta, V., Lenhard-Schuller, R., and Tonegawa, S.,** Characterization of a mouse DNA clone containing an immunoglobulin variable region gene, *Nucleic Acid Res.*, 5, 1779, 1978.
96. **Kasamatsu, H., Robberson, D. L., and Vinograd, J.,** A novel closed-circular mitochondrial DNA with properties of a replicating intermediate, *Proc. Natl. Acad. Sci. U.S.A.*, 68, 2252, 1971.
97. **Arnberg, A., Van Bruggen, E. F. J., Ter Schegget, J., and Borst, P.,** The presence of DNA molecules with a displacement loop in standard mitochondrial DNA preparations, *Biochim. Biophys. Acta*, 246, 353, 1971.
98. **Bick, M. D. and Thomas, C. A., Jr.,** A new method for mapping nucleic acid sequence homology by electron microscopy, *Nucleic Acid Res.*, 2, 509, 1975.
99. **Broude, N. E. and Budowsky, E. I.,** The reaction of glyoxal with nucleic acid components. V. Denaturation of DNa under the action of glyoxal, *Biochim. Biophys. Acta*, 294, 378, 1973.
100. **Hyman, R. W. and Summers, W.,** Isolation and physical mapping of T7 gene 1 messenger RNA, *J. Mol. Biol.*, 71, 573, 1972.
101. **Morrow, J. F., Berg, P., Kelly, T. J., Jr., and Lewis, A. M., Jr.,** Mapping of simian virus 40 early functions on the viral chromosome, *J. Virol.* 12, 653, 1973.
102. **Ferguson, J. and Davis, R. W.,** An electron microscopic method for studying and mapping the region of weak sequence homology between simian virus 40 and polyoma DNAs, *J. Mol. Biol.*, 94, 135, 1975.
103. **Broker, T. R., Soll, L., and Chow, L. T.,** Underwound loops in self-renatured DNA can be diagnostic of inverted duplications and translocated sequences, *J. Mol. Biol.*, 113, 579, 1977.
104. **Stettler, U. H., Weber, H., Koller, T., and Weissmann, C.,** Preparation and characterization of form V DNA, the duplex DNA resulting from association of complementary, circular single-stranded DNA, *J. Mol. Biol.*, 131, 21, 1979.
105. **Wellauer, P. K. and Dawid, I. B.,** Secondary structure maps of RNA: processing of HeLa ribosomal RNA, *Proc. Natl. Acad. Sci. U.S.A.*, 70, 2827, 1973.
106. **Wellauer, P. K. and Dawid, I. B.,** Secondary structure maps of ribosomal RNA and DNA. I. Processing of *Xenopus laevis* ribosomal RNA and structure of single-stranded ribosomal DNA, *J. Mol. Biol.*, 89, 379, 1974.
107. **Wellauer, P. and Reeder, R. H.,** A comparison of the structural organization of amplified ribosomal DNA from *Xenopus mulleri* and *Xenopus laevis*, *J. Mol. Biol.*, 94, 151, 1975.
108. **Hsu, M. and Jelinek, W. R.,** Mapping of inverted repeated DNA sequences within the genome of simian virus 40, *Proc. Natl. Acad. Sci. U.S.A.*, 74, 1631, 1977.
109. **Wu, M., Manor, H., and Davidson, N.,** Secondary structures in polyoma DNA, *J. Virol.*, 32, 334, 1979.
110. **Shen, C. J. and Hearst, J. E.,** Psoralen-crosslinked secondary structure map of single-stranded virus DNA, *Proc. Natl. Acad. Sci. U.S.A.*, 73, 2649, 1976.
111. **Hanson, C. V., Shen, C. J., and Hearst, J. E.,** Cross-linking of DNA in situ as a probe for chromatin structure, *Science*, 193, 62, 1976.
112. **Cech, T. R., and Pardue, M. L.,** Electron microscopy of DNA crosslinked with trimethylpsoralen: test of the secondary structure of eukaryotic inverted repeat sequences, *Proc. Natl. Acad. Sci. U.S.A.*, 73, 2644, 1976.
113. **White, R. L. and Hogness, D. S.,** R-loop mapping of the 18S and 28S sequences in the long and short repeating units of *Drosophila melanogaster* rDNA, *Cell*, 10, 177, 1977.
114. **Thomas, M., White, R. L., and Davis, R. W.,** Hybridization of RNA to double-stranded DNA: formation of R-loops, *Proc. Natl. Acad. Sci. U.S.A.*, 73, 2294, 1976.
115. **McCarthy, B. J. and Church, R. B.,** The specificity of molecular hybridization reactions, *Annu. Rev. Biochem.*, 39, 131, 1970.
116. **Birnstiel, M. L., Sells, B. H., and Purdom, I. F.,** Kinetic complexity of RNA molecules, *J. Mol. Biol.*, 63, 21, 1972.
117. **Casey, J. and Davidson, N.,** Rates of formation and thermal stabilities of RNA:DNA and DNA:DNA duplexes at high concentrations of formamide, *Nucleic Acid Res.*, 4, 1539, 1977.
118. **Chien, Y. and Davidson, N.,** RNA:DNA hybrids are more stable than DNA:DNA duplexes in concentrated perchlorate and trichloracetate solutions, *Nucleic Acid Res.*, 5, 1627, 1979.
119. **Tonegawa, S., Brack, C., Hozumi, N., and Schuller, R.,** Cloning of an immunoglobulin variable region gene from mouse embryo, *Proc. Natl. Acad. Sci. U.S.A.*, 74, 3518, 1977.
120. **Tonegawa, S., Maxam, A. M., Tizard, R., Bernard, O., and Gilbert, W.,** Sequence of a mouse germline gene for a variable region of an immunoglobulin light chain, *Proc. Natl. Acad. Sci. U.S.A.*, 75, 1485, 1978.
121. **Holmes, D. S., Cohn, R. H., Kedes, L. H., and Davidson, N.,** Positions of sea urchin *Strongylocentrotus purpuratus*) histone genes relative to restriction endonuclease sites on the chimeric plasmids pSp2 and pSp17, *Biochemistry*, 16, 1504, 1977.

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122. **Sakano, H., Rogers, J. H., Hüppi, K., Brack, C., Traunecker, A., Maki, R., Wall, R., and Tonegawa, S.,** Domains and the hinge region of an immunoglobulin heavy chain are encoded in separate DNA segments, *Nature (London)*, 277, 627, 1979.
123. **Chow, L. T., Gelinas, R. E., Broker, T. R., and Roberts, R. J.,** An amazing sequence arrangement at the 5′ ends of Adenovirus 2 messenger RNA, *Cell*, 12, 1, 1977.
124. **Chow L. T. and Broker, T. R.,** The spliced structures of Adenovirus 2 fiber message and the other late mRNAs, *Cell*, 15, 497, 1978.
125. **Broker, T. R., Chow, L. T., Dunn, A. R., Gelinas, R. E., Hassell, J. A., Klessig, D. F., Lewis, J. B., Roberts, R. J., and Zain, B. S.,** Adenovirus-2 messengers — an example of baroque molecular architecture, *Cold Spring Harbor Symp. Quant. Biol.*, 42, 531, 1977.
126. **Chow, L. T., Broker, T. R., and Lewis, J. B.,** Complex splicing patterns of RNAs from the early regions of Adenovirus-2, *J. Mol. Biol.*, 134, 265, 1979.
127. **Kilpatrick, B. A., Gelinas, R. E., Broker, T. R., and Chow, L. T.,** Comparison of late mRNA splicing among class B and class C Adenovirus, *J. Virol.*, 30, 899, 1979.
128. **Hyman, R. W.,** Physical mapping of T7 messenger RNA, *J. Mol. Biol.*, 61, 369, 1971.
129. **Deonier, R. C., Ohtsubo, E., Lee, H. J., and Davidson, N.,** Electron microscope heteroduplex studies of sequence relations among plasmids of *Escherichia coli*, *J. Mol. Biol.*, 89, 619, 1974.
130. **Ohtsubo, E., Soll, L., Deonier, R. C., Lee, H. J., and Davidson, N.,** Electron microscope heteroduplex studies of sequence relations among plasmids of *Escherichia coli*. VIII. The structure of bacteriophage ϕ80d,ilv⁺-su⁺7, including the mapping of the ribosomal RNA genes, *J. Mol. Biol.*, 89, 631, 1974.
131. **Brack, C.,** unpublished results, 1979.
132. **Kaback, D. B., Angerer, L. M., and Davidson, N.,** Improved methods for the formation and stabilization of R-loops, *Nucleic Acid Res.*, 6, 2499, 1979.
133. **Chow, L. T., Roberts, J. M., Lewis, J. B., and Broker, T. R.,** A map of cytoplasmic RNA transcripts from lytic Adenovirus type 2, determined by electron microscopy of RNA:DNA hybrids, *Cell*, 11, 819, 1977.
134. **Westphal, H., Meyer, J., and Maizel, J. V., Jr.,** Mapping of Adenovirus messenger RNA by electron microscopy, *Proc. Natl. Acad. Sci. U.S.A.*, 73, 2069, 1976.
135. **Meyer, J., Neuwald, P.D., Lai, S., Maizel, J. V., Jr., and Westphal, H.,** Electron microscopy of late Adenovirus type 2 mRNA hybridized to double-stranded viral DNA, *J. Virol.*, 21, 1010, 1977.
136. **Berget, S. M., Moore, C., and Sharp, P.A.,** Spliced segments at the 5′ terminus of Adenovirus 2 late RNA, *Proc. Natl. Acad. Sci. U.S.A.*, 74, 3171, 1977.
137. **Brack, C. and Tonegawa, S.,** Variable and constant parts of the immunoglobulin light chain gene of a mouse myeloma cell are 1250 nontranslated bases apart, *Proc. Natl. Acad. Sci. U.S.A.*, 74, 5652, 1977.
138. **Bender, W. and Davidson, N.,** Mapping of poly(A) sequences in the electron microscope reveals unusual structure of type C oncornavirus RNA molecules, *Cell*, 7, 595, 1976.
139. **Early, P. W., Davis, M. M., Kaback, D. B., Davidson, N., and Hood, L.,** Immunoglobulin heavy chain gene organization in mice: analysis of a myeloma genomic clone containing variable and α constant regions, *Proc. Natl. Acad. Sci. U.S.A.*, 76, 857, 1979.
140. **Bender, W., Davidson, N., Kindle, K. L., Taylor, W. C., Silverman, M., and Firtel, R. A.,** The structure of M6, a recombinant plasmid containing Dictyostelium DNA homologous to actin messenger RNA, *Cell*, 15, 779, 1978.
141. **Tilghman, S. M., Tiemeier, D. C., Seidman, J. G., Peterlin, B. M., Sullivan, M., Maizel, J. V., and Leder, P.,** Intervening sequence of DNA identified in the structural portion of a mouse β-globin gene, *Proc. Natl. Acad. Sci. U.S.A.*, 75, 725, 1978.
142. **Wu, M. and Davidson, N.,** A technique for mapping transfer RNA genes by electron microscopy of hybrids of ferritin-labeled transfer RNA and DNA: the ϕ 80hpsu⁺; $_m$-system, *J. Mol. Biol.*, 78, 1, 1973.
143. **Angerer, L., Davidson, N., Murphy, W., Lynch, D., and Attardi, G.,** An electron microscope study of the relative positions of the 4S and ribosomal RNA genes in HeLa cell mitochondrial DNA, *Cell*, 9, 81, 1976.
144. **Niyogi, S. K. and Thomas, C. A., Jr.,** The specific association of ribooligonucleotides of known chain length with denatured DNA, *Biochem. Biophys. Res. Comm.*, 26, 51, 1967.
145. **Niyogi, S. K.,** The influence of chain length and base composition on the specific association of oligoribonucleotides with denatured deoxyribonucleic acid, *J. Biol. Chem.*, 244, 1576, 1969.
146. **Bernard, O., Hozumi, N., and Tonegawa, S.,** Sequences of mouse immunoglobulin light chain genes before and after somatic changes, *Cell*, 15, 1133, 1978.
147. **Sakano, H., Hüppi, K., Heinrich, G., and Tonegawa, S.,** Sequences at the somatic recombination sites of immunoglobulin light-chain genes, *Nature (London)*, 280, 288, 1979.

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148. **Akusjärvi, G. and Pettersson, U.**, Sequence analysis of Adenovirus DNA. IV. The genomic sequences encoding the common tripartite leader of late Adenovirus messenger RNA, *J. Mol. Biol.*, 134, 143, 1979.
149. **Sharp, P. A., Cohen, S., nd Davidson, N.**, Electron microscope heteroduplex studies of sequence relations among plasmids of *Escherichia coli*. II. Structure of drug resistance (R) factors and F factors, *J. Mol. Biol.*, 75, 235, 1973.
150. **Chow, L. T. and Davidson, N.**, Electron microscope study of the structure of the *Bacillus subtilis* prophages, SPO2 and ϕ105, *J. Mol. Biol.*, 75, 257, 1973.
151. **Max, E. E., Seidman, J. G., and Leder, P.**, Sequences of five potential recombination sites encoded close to an immunoglobulin κ constant region gene, *Proc. Natl. Acad. Sci. U.S.A.*, 76, 3450, 1979.
152. **Maki, R., Traunecker, A., Sakano, H., Roeder, W., and Tonegawa, S.**, Exxon shuffling generates an immunoglobulin heavy chain gene, *Proc. Natl. Acad. Sci, U.S.A.*, 77, 2138, 1980.
153. **Wu, M., Davidson, N., Attardi, G., and Aloni, Y.**, Expression of the mitochondrial genome in HeLa cells. XIV. The relative positions of the 4S RNA genes and of the ribosomal RNA genes in mitochondrial DNA, *J. Mol. Biol.*, 71, 81, 1972.
154. **Broker, T. R., Angerer, L. M., Yen, P. H., Hershey, N. D., and Davidson, N.**, Electron microscopic visualization of tRNA genes with ferritin-avidin:biotin labels, *Nucleic Acid Res.*, 5, 365, 1978.
155. **Sodja, A. and Davidson, N.**, Gene mapping and gene enrichment by the avidin-biotin interaction: use of cytochrome-c as a polyamine bridge, *Nucleic Acid Res.*, 5, 385, 1978.
156. **Manning, J., Hershey, N. D., Broker, T. R., Pellegrini, M., Mitchell, H. K., and Davidson, N.**, A new method of in situ hybridization, *Chromosoma*, 53, 107, 1975.
157. **Pellegrini, M., Holmes, D. S., and Manning, J.**, Application of the avidin-biotin method of gene enrichment to the isolation of long double-stranded DNA containing specific gene sequences, *Nucleic Acid Res.*, 4, 2961, 1977.
158. **Richards, O. C., Ehrenfeld, E., and Manning, J.**, Strand-specific attachment of avidin-spheres to double-stranded poliovirus RNA, *Proc. Natl. Acad. Sci. U.S.A.*, 76, 676, 1979.
159. **Reed, S. I., Ferguson, J., Davis, R. W., and Stark, G. R.**, T antigen binds to simian virus 40 DNA at the origin of replication, *Proc. Natl. Acad. Sci. U.S.A.*, 72, 1605, 1975
160. **Wu, M. and Davidson, N.**, An electron microscopic method for the mapping of proteins attached to nucleic acids, *Nucleic Acid Res.*, 5, 2825, 1978.
161. **Wu, M., Davidson, N., and Wimmer, E.**, An electron microscope study of the proteins attached to polio virus RNA and its replicative form (RF), *Nucleic Acid Res.*, 5, 4711, 1978.
162. **Wu, M., Hyman, R. W., and Davidson, N.**, Electron microscopic mapping of protein bound to herpes simplex virus DNA, *Nucleic Acid Res.*, 6, 3427, 1979.
163. **Lescure, B. and Yaniv, M.**, Denaturation map of polyoma DNA, *J. Virol.*, 16, 720, 1975.
164. **Stüber, D., Delius, H., and Bujard, H.**, Electron microscopic analysis of in vitro transcriptional complexes: mapping of promoters of the coliphage T5 genome, *Mol. Gen. Genet.*, 166, 141, 1978.
165. **Botchan, P.**, An electron microscopic comparison of transcription on linear and superhelical DNA, *J. Mol. Biol.*, 105, 161, 1976.
166. **Brack, C.**, Electron microscopic analysis of transcription: mapping of initiation sites and direction of transcription, *Proc. Natl. Acad. Sci. U.S.A.*, 76, 3164, 1979.
167. **Davis, R. W. and Hyman, R. W.**, Physical locations of the in vitro RNA initiation site and termination sites of T7M DNA, *Cold Spring Harbor Symp. Quant. Biol.*, 35, 269, 1970.
168. **Alberts, B. M. and Frey, L.**, T4 bacteriophage gene 32: a structural protein in the replication and recombination of DNA, *Nature (London)*, 227, 1313, 1970.
169. **Brack, C. and Gehring, W.**, unpublished results, 1979.
170. **Miller, O. L., Hamkalo, B. A., and Thomas, C. A., Jr.**, Visualization of bacterial genes in action, *Science*, 169, 392, 1970.
171. **McKnight, S. L. and Miller, O. L., Jr.**, Electron microscopic analysis of chromatin replication in the cellular blastoderm *Drosophila melanogaster* embryo, *Cell*, 12, 795, 1977.
172. **Miller, O. L., Jr. and Bakken, A. H.**, Morphological studies of transcription, *Acta Endocrinol. (Copenhagen) Suppl.*, 168, 155, 1972.
173. **Meyer, G. F. and Hennig, W.**, The nucleolus in primary spermatocytes of *Drosophila hydei*, *Chromosoma*, 46, 121, 1974.
174. **Scheer, U., Trendelenburg, M. F., and Franke, W. W.**, Transcription of ribosomal RNA cistrons, *Exp. Cell. Res.*, 80, 175, 1973.
175. **Franke, W. W., Scheer, U., Spring, H., Trendelenburg, M. R., and Krohne, G.**, Morphology of transcriptional units of rDNA, *Exp. Cell. Res.*, 100, 233, 1976.
176. **Glätzer, K. H.**, Visualization of gene transcription in spermatocytes of *Drosophila hydei*, *Chromosoma*, 53, 371, 1975.
177. **Foe, V. E., Wilkinson, L. E., and Laird, C. D.**, Comparative organization of active transcription units in *Oncopeltus fasciatus*, *Cell*, 9, 131, 1976.

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178. Chromatin, *Cold Spring Harbor Symp. Quant. Biol.*, 42, 1977,
179. **Trendelenburg, M. F. and Gurdon, J. B.**, Transcription of cloned Xenopus ribosomal genes visualized after injection into oocyte nuclei, *Nature (London)*, 276, 292, 1978.
180. **Trendelenburg, M. F., Zentgraf, H., Franke, W. W., and Gurdon, J. B.**, Transcription patterns of amplified Dytiscus genes coding for ribosomal RNA after injection into Xenopus oocyte nuclei, *Proc. Natl. Acad. Sci. U.S.A.*, 75, 3791, 1978.
181. **Mertz, J. E. and Gurdon, J. B.**, Purified DNAs are transcribed after microinjection into Xenopus oocytes, *Proc. Natl. Acad. Sci. U.S.A.*, 74, 1502, 1977.
182. **Brown, D. D. and Gurdon, J. B.**, High-fidelity transcription of 5S DNA injected into Xenopus oocytes, *Proc. Natl. Acad. Sci. U.S.A.*, 74, 2064, 1977.
183. **Zentgraf, H., Trendelenburg, M. F., Spring, H., Scheer, U., Franke, W. W., Müller, U., Drury, K. C., and Rungger, D.**, Mitochondrial DNA arranged into chromatin-like structures after injection into amphibian oocyte nuclei, *Exp. Cell Res.*, 122, 363, 1979.
184. **Griffith, J., Huberman, J. A., and Kornberg, A.**, Electron microscopy of DNA polymerase bound to DNA, *J. Mol. Biol.*, 55, 209, 1971.
185. **Bickle, T. A., Brack, C., and Yuan, R.**, ATP-induced conformational changes in the restriction endonuclease from *Escherichia coli* K-12, *Proc. Natl. Acad. Sci. U.S.A.*, 75, 3099, 1978.
186. **Weideli, H.-J., Brack, C., and Gehring, W.**, Characterization of *Drosophila* DNA binding protein DB2: demonstration of its sequence specific interaction with DNA, *Proc. Natl. Acad. Sci. U.S.A.*,
187. **Jack, R., Brack, C., and Gehring, W.**, unpublished results.,1979
188. **Olins, A. L. and Olins, D. E.**, Spheroid chromatin units (Nu bodies), *Science*, 183, 330, 1974.
189. **Alberts, B., Frey, L., and Delius, H.**, Isolation and characterization of gene 5 protein of filamentous bacterial viruses, *J. Mol. Biol.*, 68, 139, 1972.
190. **Marcoli, R., Pirrotta, V., and Franklin, R. M.**, Interaction between bacteriophage PM2 protein IV and DNA, *J. Mol. Biol.*, 131, 107, 1979.
191. **Lenhard-Schuller, R., Hohn, B., Brack, C., Hirama, M., and Tonegawa, S.**, DNA clones containing mouse immunoglobulin κ chain genes isolated by in vitro packaging into phage λ coats, *Proc. Natl. Acad. Sci. U.S.A.*, 75, 4709, 1978.
192. **Murray, N. E. and Kelley, W. S.**, Characterization of λpolA transducing phages; effective expression of the *E. coli* polA gene, *Mol. Gen. Genet.*, 175, 77, 1979.
193. **Artavanis-Tsakonas, S., Schedl, P., Mirault, M.-E., Moran, L., and Lis, J.**, Genes for the 70,000 dalton heat shock protein in two cloned *D. melanogaster* DNA segments, *Cell*, 17, 9, 1979.
194. **Griffith, J.**, personal communication., 1980
195. **Portmann, R.**, personal communication., 1979.
196. **Koller, T.**, personal communication., 1979
197. **Dubochet, J.**, personal communication., 1979
198. **Broker, T. and Chow, L.**, personal communication.
199. **Meyer, J., Carlemalm, M., Lida, S., and Arber, W.**, manuscriipt in preparation.
200. **Brack, C.**, unpublished data., 1980.
201. **Yuan, R., Hamilton, D. L., and Burckhardt, J.**, DNA translocation by the restriction enzyme from *E. coli* K, *Cell*, 20, 237, 1980.

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