Hybridization of Nuclear Matrix Attached Deoxyribonucleic Acid Fragments†

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ABSTRACT: Annealing studies were performed on DNA fragments associated with rat and mouse liver interphase nuclear matrix and the metaphase scaffold of Chinese hamster DON cells. Matrix and scaffold bound DNA fragments, reassociated with an excess of total genomic DNA, displayed kinetics virtually identical with total nuclear DNA probes. Moreover, both the extent and kinetics of these hybridizations were independent of the matrix DNA fragment size (<350–5000 base pairs) and the method of nuclease digestion used in their preparation (DNase I, micrococcal nuclease or endogenous digestion). The repetitive DNA component of the matrix DNA was examined by reacting discrete sizes of matrix DNA fragments (<350–5000 base pairs) from mouse liver with a library of cloned repetitive sequence DNA fragments which included mouse major satellite sequences. Our results demonstrate that short DNA fragments anchored to the nuclear matrix contain these cloned sequences in similar proportion to total nuclear DNA and, when viewed in light of the annealing results, indicate that matrix DNA is not enriched in either repetitive or unique sequences. Furthermore, the matrix DNA fragments appear to contain the entire sequence complexity of the genome. Finally, we hybridized both matrix and total nuclear DNA fragments with cDNA to total nuclear polyadenylated RNA. The kinetics and extent of hybridization indicate that most, if not all, of the actively transcribed DNA sequences are present in similar concentrations. We conclude that in the overall organization of eukaryotic DNA within the nucleus, the repeating domains or loops which have been demonstrated by a number of investigators are not anchored at specific attachment sequences in interphase cells or during mitosis. These findings are discussed with regard to current concepts of eukaryotic DNA loop organization.

Several lines of evidence indicate that eukaryotic DNA is arranged in the cell nucleus in repeating domains or "loops" with an average size of 60–200 kilobases (Cook & Brazell, 1975, 1976; Benyajati & Worcel, 1976; Hartwig, 1978; Igo-Kemenes & Zachau, 1978; Dijkwel et al., 1979; Razin et al., 1979; Pardoll et al., 1980; Berezney & Buchholtz, 1981a). These putative DNA loops are highly supercoiled, indicating that they are constrained or fixed at both ends (Ide et al., 1975; Cook & Brazell, 1975, 1976; Benyajati & Worcel, 1976; Hartwig, 1978; Vogelstein et al., 1980). Moreover, the domains of supercoiled DNA persist and are of similar size throughout the cell cycle including mitosis (Warren & Cook, 1978). A repeating loop organization for eukaryotic DNA has potentially important implications for a variety of nuclear processes including the three-dimensional spatial arrangement of DNA in the nucleus, DNA replication, transcription, and chromosome condensation and decondensation during mitosis and meiosis.

Considerable evidence suggests that the repeating DNA loops are constrained by attachment to nonhistone protein core structures (Ide et al., 1975; Adolph et al., 1977; Laemmli et al., 1978; Razin et al., 1979; Berezney & Buchholtz, 1981a). Estimates of the number of DNA attachment sites per nucleus have varied from 60000 to 125000 depending on the cell type in question (Razin et al., 1979; Berezney & Buchholtz, 1981a). In addition, it has been suggested that the small fragments of DNA which remain as relatively intractable constituents of the nuclear protein matrix (Berezney & Coffey, 1974, 1977; Berezney, 1979a), and the metaphase chromosome scaffold (Adolph et al., 1977; Laemmli et al., 1978), represent the basal attachment fragments for the loops (Laemmli et al., 1978; Jeppesen & Bankier, 1979; Razin et al., 1979; Berezney & Buchholtz, 1981a). Consistent with this interpretation, our laboratory has recently isolated nuclear matrices while avoiding significant cleavage of the nuclear DNA (Berezney & Buchholtz, 1981b). Under these conditions, most of the high molecular weight DNA remains associated with the matrix structures, and whole mount electron microscopy has further demonstrated a repeating loop organization for this DNA (Berezney et al., 1980, 1981a; R. Berezney and A. J. Siegel, unpublished results).

A fundamental question to consider is whether there are specific attachment sequences which anchor the DNA at the matrix attachment sites. Clearly, such a family of sequences could provide a mechanism for the propagation of an exact spatial ordering of DNA loops from one cell generation to another. Although several investigations have addressed this question, the results appear contradictory (Razin et al., 1978, 1979; Dvorkin & Vanyushin, 1979; Jeppesen & Bankier, 1979; Pardoll & Vogelstein, 1980). In our attempt to answer this question, we examined the sequence of matrix-attached DNA fragments from several points of view including reassociation kinetics, cDNA hybridization, and screening for specific sequences by using cloned repetitive DNA fragments. Our data demonstrate that the overall population of small DNA fragments attached to both interphase nuclear matrix and the metaphase scaffold is random in sequence with no apparent enrichment for a specific "attachment site sequence".

Materials and Methods

Nuclear and Matrix Isolation. Nuclei and nuclear matrices were prepared from rat and mouse liver on the basis of previously described procedures (Berezney & Buchholtz, 1981a; Berezney & Coffey, 1977). Fresh or frozen livers were minced and homogenized with 10 up and down strokes in a Potter–Elvehjem homogenizer operating at 1000 rpm (Arthur J. Thomas Co.) and containing approximately 4 volumes of su-

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crose TM (0.25 M sucrose, 10 mM Tris,[1] pH 7.4, and 5 mM $MgCl_2$). Nuclei were initially separated by low-speed centrifugation (1000*g*, 10 min, Sorvall-Du Pont HB-4 rotor). The crude nuclear pellets were mixed with 2.2 M sucrose TM buffer to a concentration of about 3×10^6 nuclei/mL and centrifuged at 80000*g* for 30 min (Beckman SW27 rotor). The pellet containing purified nuclei was then resuspended gently and washed once with sucrose TM buffer prior to matrix isolation. All procedures were performed at 0–4 °C. In addition, 1 mM PMSF (phenylmethanesulfonyl fluoride, Sigma) and 0.1 mM sodium tetrathionate (ICN Pharmaceuticals) were added in most experiments to the nuclear isolation buffers when the nuclei were subsequently digested with the exogenously added nucleases (DNase I or micrococcal nuclease). If the nuclei were endogenously digested (see below), both PMSF and tetrathionate were omitted during nuclei isolation since we have found that both these reagents substantially reduce the endogenous DNase activity of isolated liver nuclei (R. Berezney, unpublished data).

Nuclei were resuspended to about 1 mg of nuclear DNA/mL in sucrose TM and digested via the endogenous nuclease activity (Hewish & Burgoyne, 1973), micrococcal nuclease (Worthington), or DNase I (Worthington) for the indicated times and temperatures. When DNase I was used, the incubation conditions that yielded matrix-attached DNA of mean fragment length of 250–300 base pairs were 40 enzyme units (Worthington) per mg of nuclear DNA for 5 min at 30 °C. Micrococcal nuclease digestion of nuclei yielded matrix-associated DNA with a typical multimeric unit distribution with 2 enzyme units (Worthington) per mg of nuclear DNA for 40 min at 30 °C. Endogenous nuclease digestion was performed for 30–120 min at 37 °C. The proportion of DNA in the monomeric peaks for the latter two types of digestion could be increased by increasing incubation time (data not shown). After digestion, the suspension was quickly chilled to 0 °C and centrifuged at 1000*g* for 10 min. When micrococcal nuclease or endogenous activity was used, the reaction was stopped by addition of EGTA to 10 mM (pH 7.4). The postdigestion supernatant was analyzed for DNA, RNA, and protein content. Digested nuclei were then resuspended in a low magnesium, low ionic strength buffer, LS (10 mM Tris, pH 7.4, and 0.2 mM $MgCl_2$), incubated for 5–10 min (0 °C) and pelleted at 1000*g* for 15 min. This procedure was repeated twice, and LS supernatants were combined for analysis. The remaining nuclear pellet was further extracted with a high salt buffer, HS (2.0 M NaCl, 10 mM Tris, pH 7.4, and 0.2 mM $MgCl_2$), in a similar fashion. Again, HS supernatants were combined for analysis. The matrix high salt resistant pellet was then rinsed once with LS and finally resuspended in a small volume of LS. Sometimes a Triton X-100 extraction (1% TX-100, 10 mM Tris, pH 7.4, and 0.2 mM $MgCl_2$) was included after the high salt extraction to remove the nuclear membrane. All extraction steps were performed with at least 20 volumes of buffer per nuclear pellet. In most experiments, 1 mM PMSF and 0.1 mM sodium tetrathionate were added to the LS and HS buffers.

Isolation of Metaphase Chromosomes and Scaffolds. Chromosomes and nuclei were isolated from a Chinese hamster cell line, DON, as previously described (Matsui et al., 1972a,b, 1979a,b; Goyanes et al., 1980). Chromosome scaffolds were prepared in essentially the same manner as the interphase matrix, except digestion of chromosomes was done exclusively with micrococcal nuclease (1 Worthington unit, A_{260}, 5 min, 37 °C) in 10 mM Tris, pH 7.5, 2 mM $CaCl_2$, 2.5 mM $MgCl_2$, and 0.5 mM PMSF, and pellets were gathered at 4000*g* for 15 min. This method of scaffold preparation was similar to previously reported procedures (Razin et al., 1979; Adolph et al., 1977; Jeppesen & Bankier, 1979).

Chemical Determinations. RNA and DNA were separated by the methods of Munro & Fleck (1965). DNA and RNA were quantitated by absorption at 260 nm and protein by the method of Lowry et al. (1951).

Isolation of DNA Fragments. DNA was directly isolated from both pellets and supernatants, though in some cases when volume became a problem, supernatants were first mixed with 2–3 volumes of ethanol (95%, –20 °C) to precipitate and concentrate the DNA. Samples, free of PMSF and tetrathionate, were resuspended in a small volume of TE (20 mM Tris, pH 7.4, and 5 mM EDTA) buffer or made 5 mM in EDTA (in the case of supernatants), digested with 50–100 μg/mL RNase A Sigma) at 0 °C for 30 min, and then warmed to 25 °C for another 30 min. The sample was then made 100 μg/mL in proteinase K (Boehringer Mannheim) and incubated at 25 °C for 30 min and subsequently at 37 °C for 30 min. The latter incubation was performed in the presence of 0.5% $NaDodSO_4$. No particulate matter could be detected at the end of these incubations. The sample was then extracted exhaustively with buffer-saturated phenol/chloroform/isoamyl alcohol (50:49:1), and the aqueous phase was precipitated by the addition of 0.1 volumes of 2 M sodium acetate, pH 5.0, and 2.5 volumes of 95% ethanol (–20 °C, 6 h). DNA was removed by centrifugation (8000*g*, 60 min, 0 °C), suspended in TE buffer, redigested, extracted, and precipitated until the $A_{260}/A_{280} \geq 2.0$. DNA solutions were then made 1 × in column buffer, CB (300 mM NaCl and 10 mM sodium acetate, pH 5.0), and chromatographed on SP50 (Pharmacia) and Chelex 100 (Bio-Rad). The void volume fraction was collected and precipitated with ethanol (at –20 °C, 12 h). The procedure yields DNA of high purity, free of contaminating RNA, protein, nucleotides, phosphorous, and divalent cations. Shearing of DNA, when necessary, was accomplished by passing the DNA solution throug a French pressure cell at 3000 psi. This yielded fragments with an average size of 800 nucleotides.

Determination of DNA Fragment Lengths. DNA samples were electrophoresed on 4.5% acrylamide–urea gels following denaturation, on alkaline agarose gels, or on acetate–EDTA buffered agarose gels when denaturation was not required. The percentage of agarose in the gels varied from 0.5% to 2.0% according to the size of fragments to be separated. For acrylamide–urea gels, samples were made 1 × in TBE buffer (10 mM Tris, pH 8.3, 3 mM EDTA, and 90 mM boric acid), heated at 100 °C for 3 min, cooled at 25 °C, combined with crystalline urea (Schwarz/Mann, Ultrapure) to ~7 M, and applied to a 4.5% acrylamide gel made 1 × in TBE and 7 M in urea (150 V/2 h). When larger fragments were to be separated, samples were made 0.03 N in NaOH and 1 mM EDTA, incubated briefly, and applied to agarose gels (0.8–1.2%) which were also 0.03 M in NaOH and 1 mM EDTA (50 mA/2–6 h). Bromocresol green was used as a marker for alkaline agarose gels, while bromophenol blue served as a marker for acrylamide and nondenaturing agarose gels. Nondenatured samples were made 1 × in EB buffer (36 mM Tris, pH 8.0, 30 mM NaH_2PO_4, and 1 mM EDTA) and applied to an agarose gel made in the same buffer. Molecular

[1] Abbreviations used: Tris, tris(hydroxymethyl)aminomethane; PMSF, phenylmethanesulfonyl fluoride; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate; bp, base pair; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid.

weight size markers were provided from *Hae*III digest products of PM2 DNA (Kovacic & Van Holde, 1977) and *Hin*dIII fragments of bacteriophage λ (Old et al., 1975). Elution of DNA fragments from gels was accomplished by incubating minced gel slices in 500 mM sodium acetate, pH 5.0, 0.1 mM EDTA, and 0.1% $NaDodSO_4$ for 24–48 h at 37 °C in a shaker bath. The eluant was then extracted and precipitated as described above.

Labeling DNA Fragments. Nick translation of double-stranded DNA fragments was performed essentially by the method of Rigby et al. (1977). Removal of "fold-back" regions after nick translation was accomplished as previously described (Britten et al., 1974). DNase I (DPFF) was purchased from Worthington Biochemicals (Freehold, NJ), and DNA polymerase I was purchased from Boehringer Mannheim (Indianapolis, IN).

Preparation of cDNA. RNA was prepared by a modification of the method of Holmes & Bonner (1973) and was essentially the same as for DNA isolation with the following exceptions. DNase I, freed of ribonuclease A activity by passage over an affinity column of 5′-[(4-aminophenyl)-phosphoryl]uridine 2′(3′)-phosphate–Sepharose (Wang & Moore, 1978; Maxwell et al., 1977), was used in place of the RNase A digestions and in the presence of 1–2 mM $MgCl_2$. Proteinase K digestions were performed only at 25 °C in the presence of 0.5% $NaDodSO_4$. Nuclei used for RNA preparation were prepared in buffers containing 10 mM vanadium nucleoside complex to inhibit RNase activity (Berger & Birkenmeier, 1979). Isolation of polyadenylated RNA and cDNA preparation was accomplished essentially as described by Toole et al. (1979).

Liquid Hybridizations. Hybridizations were carried out according to the following protocol. The experimental probe (e.g., ^3H-labeled matrix DNA) was mixed with an equal number of counts of control probe (e.g., ^{32}P-labeled total nuclear DNA) in the presence of a 10000-fold excess of driver DNA (same as control probe). The reaction mixture was heated to 100 °C for 5 min and cooled to 65 °C, and hybridization was initiated with the addition of 0.2 volume of 5 M NaCl, 5 mM EDTA, and 100 mM Hepes, pH 7.4. The reaction was generally run at 7–10 mg/mL driver DNA in 50–100 μL volume. The amount of double-stranded DNA formed with time was measured by the S1 nuclease method (Leong et al., 1972) and the hydroxylapatite method (Britten et al., 1974; Hastie & Bishop, 1976). Reassociation curves were normalized such that the maximum extent of hybridization of each probe in the reaction mixture represented 100%. All other values for individual probes were then calculated relative to their maximum values.

Filtering Hybridizations. Hybridization of probes to cloned repetitive gene fragments was carried out by the method of Grunstein & Hogness (1975). Clones were grown on nitrocellulose filters (S&S, BA85) and lysed by gentle washing with four changes each of (A) 50 mM Tris, pH 8.0, 50 mM EDTA, 25% sucrose, and 1.5 g/mL lysozyme (Sigma) at 4 °C, (B) 0.5 N NaOH and 0.2% Triton X-100 at 25 °C, (C) 1 N NaOH at 25 °C, (D) 1 M Tris, pH 7.5, at 25 °C, and (E) 0.15 M NaCl and 0.1 M Tris, pH 7.5, at 25 °C. Filters were dried and baked at 80 °C for 4 h in vacuo and then prehybridized for 4 h at 65 °C with 10 mL of a solution containing 4 × SSC (SSC, 0.15 M NaCl and 0.3 M sodium citrate), 1 × Denhardt's solution (Denhardt, 1966), 0.1 M phosphate buffer, pH 7.4, 1 g of dextran sulfate, and 1.0 mg/mL denatured salmon sperm DNA. Hybridization was accomplished by adding $\sim 1 \times 10^7$ cpm of ^{32}P-labeled probe and incubating 18–24 h at 65 °C with shaking. Excess ^{32}P-labeled probe was then washed away with exhaustive changes of 2 × SSC (65 °C). The filters were dried and exposed to autoradiography (Kodak XR-5 X-ray film).

Cloning of Mouse Repetitive DNA. Mouse liver DNA was renatured to C_0t 0.03 in 0.18 M NaCl buffer at 70 °C and passed through hydroxylapatite to remove fold-back DNA and to reduce the content of satellite DNA. The single-stranded fraction recovered from hydroxylapatite was then renatured to C_0t 30 in 1 M NaCl buffer at 70 °C and treated with S1 nuclease to produce a population of blunt-ended repetitive DNA duplexes free of flanking single copy sequences. These molecules were tailed with (dC) residues, inserted into plasmid PBR322 at the *Pst*1 site by the method of Villa-Komaroff et al. (1978), and transformed into *E. coli* strain λ1776. Full details of this clone library will be published elsewhere (D. Pietras, K. Gross, and N. D. Hastie, unpublished results). Briefly, approximately 125 ampicillin-sensitive, tetracycline-resistant clones were obtained which contain mouse repetitive DNA inserts. The repetition frequency, genomic organization, and transcriptional products of a number of these sequences have been characterized. All work was performed according to NIH guidelines.

Results

Generation of Nuclear Matrix Attached DNA Fragments. During nuclear matrix isolation, DNA is released from the predigested nuclei in two basic steps: the low magnesium, low ionic strength (LS) and the high salt (HS) washes (Berezney & Coffey, 1974, 1977; see Materials and Methods). The bulk of the nuclear DNA is released during the LS washes (75–80% of total DNA) and is consequently termed the low salt soluble or *LS DNA*. Another 18–24% is removed by the high salt washes and is termed high salt soluble or *HS DNA*. The small amount of DNA (1–2%) which remains tightly bound to the high salt resistant nuclear matrix is called the *matrix-attached* or *matrix DNA*.

Since the purpose of this study was to determine if any class of sequences was enriched at or near the matrix attachment site, we needed to look at DNA which contained sequences closer to and farther away from the matrix. It has previously been found that the level of nuclease digestion prior to matrix isolation determines both the size and relative recovery of DNA in the matrix (Razin et al., 1979; Berezney et al., 1981b). Figure 1 documents the progressive decrease in the size of the matrix-attached DNA fragments with increasing time of endogenous digestion in both rat and mouse liver nuclei. Similar results were obtained with micrococcal nuclease and DNase I digestion, although the latter did not give the characteristic multimer pattern. The DNA washed from the nuclei during low and high salt extractions also have these patterns (data not shown). It follows that if there are a set number of attachment sites within the nucleus, which recent evidence suggests (Razin et al., 1979; Berezney & Buchholtz, 1981a), then the smaller matrix DNA fragments generated by the digestion procedure will represent those sequences closest to the attachment site, while those farther away will be in the larger DNA fragments. An enhancement of a specific attachment sequence, if such a sequence is large enough, would likely be detected during hybridization of the matrix-attached DNA with total nuclear DNA and reflected even more so in matrix DNA reassociation kinetics.

Matrix-Attached Fragments Are Not Enriched in Repetitive or Unique DNA Sequences. We isolated, by elution from nondenaturing agarose gels, those DNA fragments (≤350 bp) which presumably lie closest to the matrix. Larger fragments

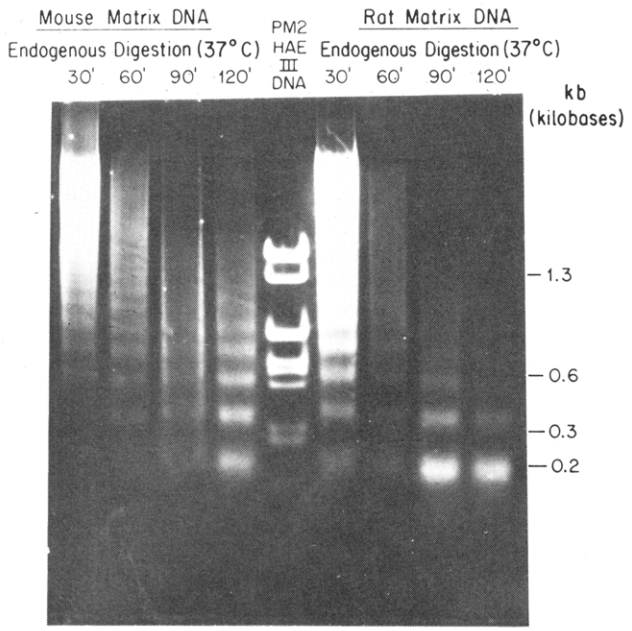

FIGURE 1: Size of matrix-attached DNA fragments following endogenous nuclease digestion of rat and mouse liver nuclei. Nuclei were digested at 37 °C for the indicated times as described under Materials and Methods. The purified matrix-attached DNA fragments for each digestion period were then electrophoresed on a 2% agarose gel with *Hae*III-restricted PM2 DNA as a size marker. The gel was stained in 0.5 μg/mL ethidium bromide for 20 min for visualization.

up to 5000 bp containing more "distal sequences" were also prepared in this manner. These fragments were nick translated and hybridized against a vast excess (10^4-fold) of sheared total nuclear DNA (average size ~800 bp). Over 20 separate preparations of total nuclear and matrix DNA probes were studied, and the results presented in Figure 2 are representative of our findings. The data have been normalized for comparison (maximum hybridization typically ranged from 80% to 90% when assayed by the hydroxylapatite method and from 60% to 70% when assayed by the S1 nuclease method). With rat liver matrix, neither the method of nuclease digestion nor the size of the attached DNA had any major effect on the hybridization curve (Figure 2). In each case, the matrix DNA curve can be superimposed over that of total DNA reassociating with itself. Mouse liver matrix DNA fragments behaved in a similar fashion, showing no real variation with isolation method or fragment size (data not shown). Figure 2B shows a very slight shift of the mouse matrix DNA curve to the right (i.e., an increased unique and decreased repetitive component), but this was not found to be significant or reproducible in several experiments. The lack of significant kinetic differences between the two, when the method of digestion and size of matrix-attached DNA (and hence concentration of any specific attachment sequence which might exist) are varied, suggests that no real difference exists in the proportions of repetitive and unique DNA between the small matrix-attached DNA fragments and total nuclear DNA.

DNAs from the LS and HS washes were also isolated and hybridized to excess sheared total nuclear DNA (Figure 3). Again, no major alterations in the C_0t curve could be demonstrated for LS DNA, HS DNA, or matrix-attached DNA when compared to total. This suggests that not only is an overall random population of fragments left in association with the matrix but also DNA is released in a random manner during the LS and HS washes. That is, no frequency class, detectable by reassociation kinetics, is preferentially lost at any given step.

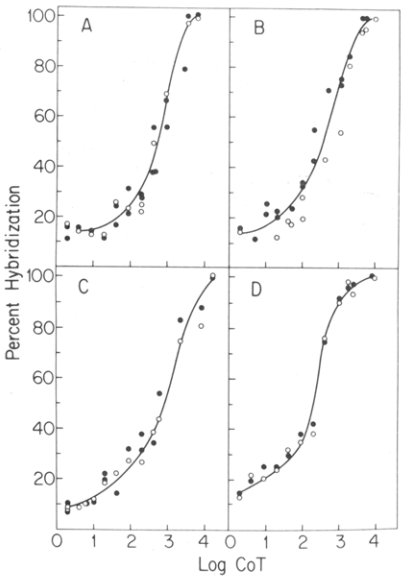

FIGURE 2: Total genomic DNA driven reassociation of matrix-attached DNA and total nuclear DNA probes. (A) Rat liver nuclear matrix-attached DNA (≤350 bp, prepared by the endogenous nuclease method) and total rat liver nuclear DNA (~400 bp) probes annealing in the presence of an excess of total rat liver nuclear DNA (≤1200 bp). Hybridization was assayed by the hydroxylapatite method and normalized as described under Materials and Methods. Maximum hybridization: total probe 87%; matrix probe 86%. (B) Mouse liver matrix-attached DNA (≤350 bp, prepared by the endogenous nuclease method) and total mouse liver DNA (~400 bp) annealing in the presence of an excess of total mouse liver nuclear DNA (~800 bp). S1 nuclease assay. Maximum hybridization: total probe 68%; matrix probe 61%. (C) Rat liver matrix-attached DNA (~800 bp), prepared by DNase I method, and total rat liver DNA (~800 bp), driven by total rat liver nuclease DNA (≤1200 bp). Hydroxylapatite assay. Maximum hybridization: total probe 95%; matrix probe 88%. (D) Rat liver matrix-attached DNA (≤350 bp, prepared by the micrococcal nuclease method) and total rat liver nuclear DNA (~400 bp) probes driven by an excess of total nuclear DNA (≤1200 bp). Hydroxylapatite assay. Maximum hybridization: total probe 85%; matrix probe 86%. Total probes (●); matrix probes (○).

Metaphase Scaffold Attached Fragments Are Not Enriched in Repetitive or Unique DNA Sequences. Isolated chromosome scaffold preparations generally contain a small amount of tightly bound DNA (Adolph et al., 1977; Jeppesen & Bankier, 1979; Razin et al., 1979). It has been suggested that the chromosome scaffold DNA fragments represent the "mitotic equivalent" of the matrix-attached DNA fragments isolated from interphase nuclei (Razin et al., 1979). Therefore, we isolated a residual chromosome scaffold fraction following a brief micrococcal nuclease digestion of Chinese hamster DON cell chromosomes (see Materials and Methods). From the total DNA fragment population associated with the chromosome scaffolds, we obtained, by gel elution, a small fragment class (≤600 bp) for hybridization analysis. Figure 4 shows the results of one experiment in which total Chinese hamster nuclear DNA was used to drive its homologous and chromosome scaffold DNA fragments. Again, the C_0t curves are practically identical, indicating that this DNA, too, contains approximately the same proportion of each sequence class as does the total DNA.

The Sequence Complexity of Matrix-Attached DNA Is Equal to Total Nuclear DNA. Next, we examined matrix driven hybridizations. These experiments were performed for two main reasons. First, a small sequence which may be enriched near the matrix attachment site could be overlooked in total DNA excess hybridization studies if other sequences are present in roughly their same normal proportions. However, if the small matrix-attached DNA fragments (e.g., ≤350

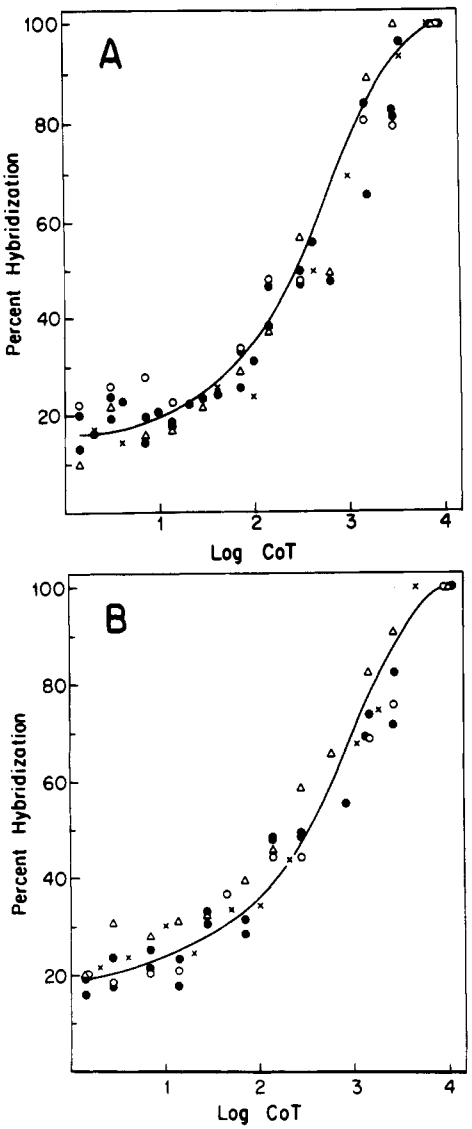

FIGURE 3: Total genomic DNA driven annealing of DNA fractions released during matrix isolation. (A) Rat liver total nuclear DNA (≤1200 bp) driving total nuclear DNA [~400 bp (●)], LS extracted DNA (○), HS extracted DNA (△), and matrix-attached DNA [≤350 bp (X)]. Maximum hybridizations were 50%, 55%, 56%, and 76% respectively, for S1 nuclease assays. DNA was digested via endogenous nuclease method. (B) Mouse liver total nuclear DNA (~800 bp) driving total nuclear DNA (~400 bp), LS DNA, HS DNA, and matrix-attached DNA (≤350 bp). Maximum hybridizations were 59%, 59%, 64%, and 61%, respectively, for S1 nuclease assays. DNA was digested via endogenous nuclease method.

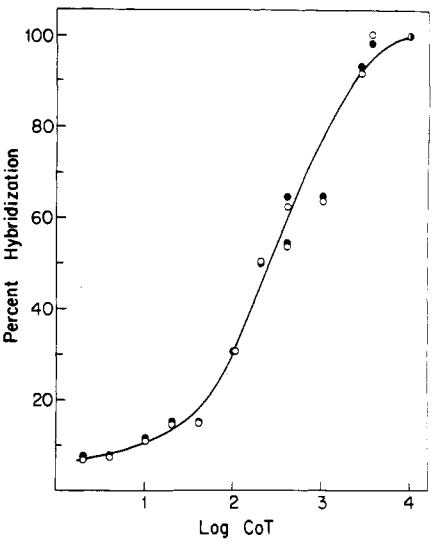

FIGURE 4: Total genomic DNA driven annealing of Chinese hamster DON cell metaphase scaffold attached DNA fragments. Metaphase chromosome scaffold structures were prepared as described under Materials and Methods by using the micrococcal nuclease method. The small scaffold-attached DNA fragments [<600 bp (○)] were isolated, labeled, and reassociated in the presence of excess total CHO nuclear DNA driver (~500 bp) and a total nuclear CHO DNA probe [~400 bp (●)]. S1 nuclease assay. Maximum hybridization: total probe 60%; scaffold-attached DNA 61%.

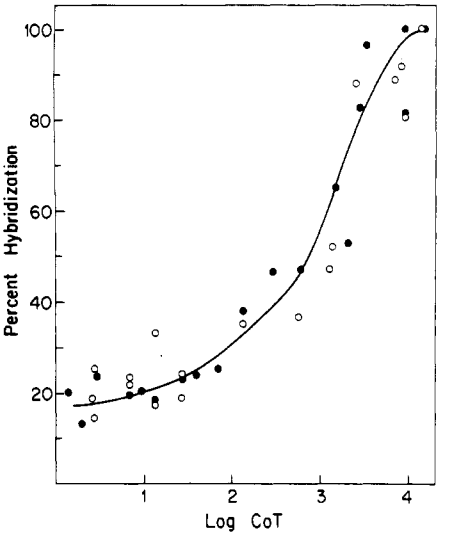

FIGURE 5: Rat liver nuclear matrix DNA driven annealing of total nuclear and matrix DNAs. Matrix probe [≤350 bp (○)] and matrix DNA driver (≤800 bp) were prepared via the endogenous nuclease method. Total nuclear DNA probe [~400 bp (●)] was from nick-translated total sheared rat liver DNA. Hydroxylapatite assay. Maximum hybridizations: total DNA 70%; matrix-attached DNA 64%.

bp) are used as a driver in the hybridization experiments, then any enriched sequence should be present in a relatively high proportion and behave as a repetitive component. Markedly different reassociation curves would then be expected for the total and matrix-attached DNA probes. Second, if specific attachment sites, which are stably transmitted from generation, exist along the eukaryotic DNA molecule, then the small DNA fragments associated with these sites would represent only a very small proportion of the total DNA complexity. Thus, the matrix-attached DNA fragments which are enriched in the specific attachment sequences would be largely incapable of driving a total nuclear DNA probe. Our results, however, demonstrated that while the absolute level of hybridization was lower than expected (maximum of 64% for matrix DNA and 70% for total DNA probes by hydroxylapatite assay), the reassociation kinetics for the two probes were virtually identical (Figure 5). The shift in both curves to the right compared to a typical total nuclear DNA excess hybridization may reflect the inability of this small DNA (average of about 140–200 bp) to drive the reaction as well as larger fragments (approximately 800 bp for total DNA excess hybridizations). In other experiments not shown, LS DNA and HS DNA also drove total DNA probe as effectively as their homologous probes.

Matrix-Attached DNA Is Not Enriched in Specific Repetitive Sequences. In contradiction to our findings, several previous studies using C_0t analysis have concluded that matrix and scaffold DNA are enriched for repetitive DNA sequences (Razin et al., 1978, 1979; Jeppesen & Bankier, 1979). With this in mind, we have designed an experiment to determine whether specific cloned repetitive sequences are enriched or depleted in matrix DNA. Figure 6 demonstrates the results

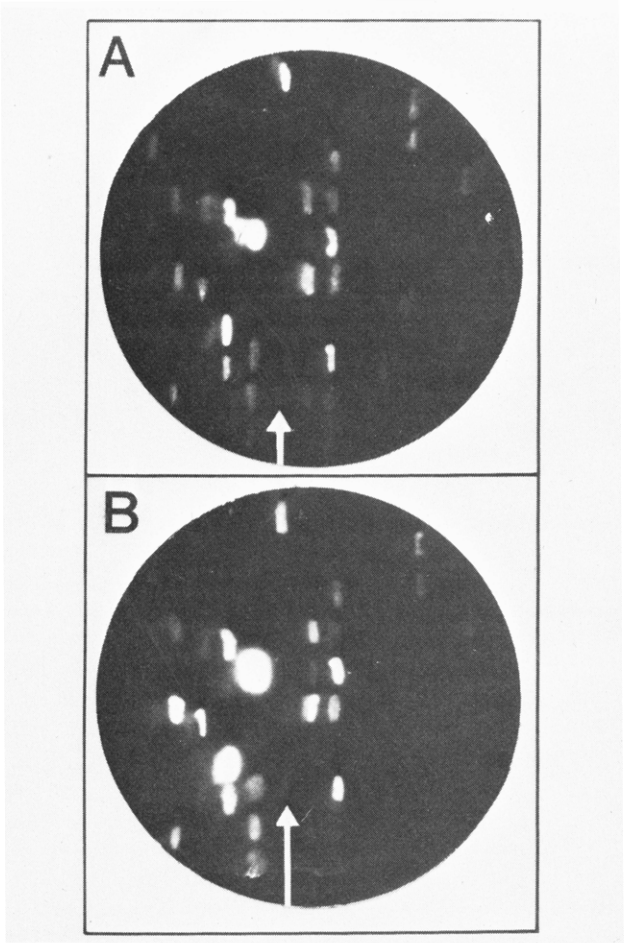

FIGURE 6: Grunstein–Hogness hybridization of (A) total nuclear mouse DNA probe (~400 bp) and (B) small nuclear matrix mouse DNA (≤350 bp) to cloned repetitive mouse DNA fragments. ^{32}P-labeled DNA probes [(5–10) × 10^7 cpm/μg) prepared by nick translation and hybridization were performed as described under Materials and Methods. Arrow indicates one clone whose position was changed from filter A to filter B to facilitate identification during handling. This figure is a direct positive print of the autoradiograph.

of two Grunstein–Hogness blots in which either labeled small (≤350 bp) matrix-attached DNA or total mouse DNA were used as probes to screen a library of cloned mouse repetitive DNA (see Materials and Methods for details). The signal intensities of total mouse DNA (Figure 6A) are roughly proportional to the clones repetition frequency in the genome. The clones giving the strongest signal are mouse major satellite (repeated about 10^6-fold), and the weakest detectable signals are obtained from clones containing mouse DNA repeated only about 500 times per genome (D. Pietras, K. Gross, K. Bennett, and N. D. Hastie, unpublished results).

Other studies have determined that differences in signal intensities caused by 3-fold variations in label concentration are easily detectable by this technique (D. Pietras, K. Gross, and N. D. Hastie, unpublished results). In the experiment shown in Figure 6, the absolute level of signal intensities for all clones was higher for the small matrix DNA probe than for the total DNA probe. This difference is likely due to any of several experimental variables such as having a greater amount of labeled probe in the small matrix DNA reaction mixture or having slightly better growth conditions for the clone colonies on the filter used to hybridize with the matrix DNA probe. More important, however, is the finding that matrix-attached and total nuclear DNA showed essentially the same reproducible pattern of relative signal intensities in this (Figures 6) and in repeat experiments. If any of these

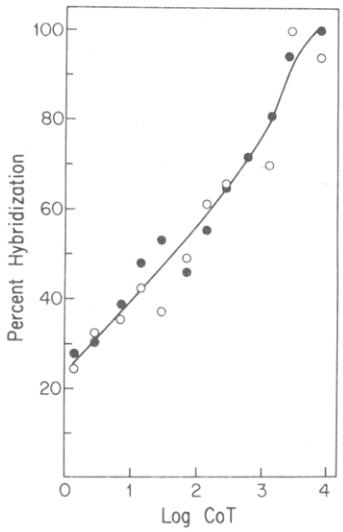

FIGURE 7: Hybridization of cDNA from total nuclear polyadenylated RNA to excess total nuclear DNA [≤1200 bp (●)] and small matrix-attached DNA fragments [≤800 bp (○)]. ^{3}H-labeled cDNA was prepared and hybridized as described under Materials and Methods. The reaction was assayed by the S1 nuclease method. Maximum hybridization: total nuclear DNA driven reaction 72%; small matrix DNA driven reaction 55%.

repetitive sequences were enriched significantly in the small matrix-attached DNA fraction they should have given much more intense signals relative to the other clones on the same filter. Also, a sequence underrepresented in a small matrix-attached probe would have given weaker relative signals with that probe as compared to total DNA. Thus, we conclude that none of these cloned sequences that are represented 500 times or more, including mouse major satellites, are enriched or depleted in small matrix-attached DNA.

The same results were observed when larger matrix-attached DNA fragments (up to 5000 bp) were used as probes (data not shown). This is especially important since statistically, a repetitive sequence repeated more than 10 000 times in the genome should be present in the clone library. These data make it highly unlikely that a specific repetitive sequence family could serve as the basis for the 60 000–125 000 matrix attachment sites.

Transcribed Sequence in Matrix-Attached DNA. We then performed an experiment to determine whether the matrix-attached fragments were enriched or depleted in an important functional subclass of the total DNA: actively transcribed sequences. cDNA prepared from total nuclear polyadenylated RNA was, therefore, hybridized with either matrix-attached or total DNA fragments as driver. Virtually identical kinetics and extent of cDNA hybridization were measured (Figure 7), indicating that transcribed sequences are found in similar proportions in matrix and total nuclear DNA.

Discussion

Recent investigations into the structural and functional relationships of the nucleus and chromosomes have resulted in an emerging model of chromatin organization which describes the dynamic interaction of protein structures and DNA (Comings, 1978; Georgiev et al., 1978; Sharper et al., 1979; Berezney, 1981; Berezney & Buchholtz, 1981a). It has been suggested that the DNA is organized into loops which are anchored at their base to protein structures, particularly the nuclear matrix and chromosome scaffold (Comings, 1978; Georgiev et al., 1978). Several studies have been directed at determining the characteristics of the DNA at the base of these loops, and an equal number of conflicting reports have ensued.

For instance, Razin et al. (1978, 1979) found in their hybridization studies that the nuclear matrix and chromosomal scaffold of mouse L cells were enriched for middle repetitive sequences when isolated following micrococcal nuclease digestion but that the axial chromosomal cores also contained highly reiterated satellite sequences when digested first with *Hin*dIII or *Eco*RI. However, the possibility that the restriction enzymes were actually selecting for satellite sequences at the attachment sites could not be ruled out (Razin et al., 1979). Although Jeppesen & Bankier (1979) also reported rapidly reassociating DNA enriched near the chromosomal cores of Chinese hamster cell chromosomes following salt extraction and digestion with micrococcal nuclease, these types of results are not universally found. Dvorkin & Vanyushin (1979), for example, described their rat liver nuclear matrix as being enriched in unique sequences on the basis of a limited study of reassociation kinetics. Even more recently, Pardoll & Vogelstein (1980) presented hybridization evidence asserting that the rat liver nuclear matrix contains an assortment of sequences of similar complexity to total nuclear DNA. In contrast, Cook & Brazell (1980) have argued for a "nonrandom arrangement" of DNA at the matrix attachment sites of HeLa nuclei.

Our results, using a variety of hybridization techniques, can be summarized in two main points: (i) the DNA immediately adjacent to the sites of interaction with the nuclear matrix or chromosomes scaffold is not enriched in repetitive or unique DNA sequences and (ii) the small matrix-attached DNA fragments, which represent less than 1% of the total nuclear DNA, have a sequence complexity equivalent to total genomic DNA. We conclude that the postulated intranuclear loop arrangement of eukaryotic DNA is not mediated by specific attachment site sequences and that the DNA closely associated with the nuclear matrix is essentially random in sequence. Our results further raise the possibility that the DNA loops are dynamic rather than statically fixed structures as previously suggested (Razin et al., 1979; Cook & Brazell, 1980). That is, the random DNA sequences at the attachment sites may reflect the overall expression of a dynamic process of DNA association–disassociation. In this manner, the number of attachment sites is preserved while the actual sites of DNA interaction are subject to change. The fact that both matrix and chromosomal attached DNAs are random in sequence composition is consistent with this dynamic loop model. For example, if the DNA were randomly arranged on the matrix just prior to mitosis, it could just as easily be represented that way during mitotic chromosomal condensation.

An alternative interpretation of our results is that the matrix-attached DNA fragments are random in sequence because these fragments, or the DNA loops from which these fragments are presumably generated, represent isolation artifacts. For instance, the similar nucleosomal repeat patterns generated in matrix-associated DNA and the bulk DNA released in LS and HS washes could be interpreted as having resulted from an artifactual precipitation of a small amount of the DNA in the matrix during the isolation procedure. While we cannot, at present, rule out the possibility of this type of artificially induced association, there is no reason to expect that certain sequences could not be arranged in nucleosomes and also be attached to the matrix structure (e.g., at, but not limited to, a site in the internucleosomal DNA). Moreover, evidence that the number of attachments (as determined by the average size of matrix DNA and the recovery of nuclear DNA in the matrix) remains constant even when more and more DNA is digested away (Razin et al., 1979; Berezney et al., 1981b) suggests that DNA association with the nuclear matrix involves a specific number of DNA molecules, independent of DNA fragment length.

Perhaps more important, however, is that previous reconstruction experiments as well as consideration of the very tight association of both nonreplicating and replicating DNA with the nuclear matrix argue strongly against an artifactual association and further suggest that the proposed attachment sites are involved in DNA replication (Berezney & Coffey, 1975, 1976; Pardoll et al., 1980; Berezney & Buchholtz, 1981a,b). A more definitive approach for defining the putative attachment sites might involve the use of cross-linking agents in vivo. The methods of van Ekelen & van Venrooiz (1981) and Mayrand & Pederson (1981), who recently studied the native interactions of hnRNA with nuclear proteins, or the method of Sinden & Pettijohn (1981), who employed a novel photobinding technique to measure repeating domains of supercoiled DNA in living *Escherichia coli* cells, may be applicable here.

While it is difficult to explain the discrepancies among previously reported results, there are several aspects of nuclear matrix systems thar are critical to consider. First, the matrix- or scaffold-attached DNA fragments used in these experiments represent a recovery of <1% of the total nuclear DNA. Mitochondria in the isolated nuclear preparations might result in significant contamination with mitochondrial DNA sequences, which, in turn, would appear as an enrichment in repetitive sequences in matrix DNA. Fortunately, the rat liver nuclei used in our studies were virtually devoid of mitochondrial contamination as evaluated by both ultrastructural analysis and enzymatic markers (J. Basler and R. Berezney, unpublished observations). Second, although the procedures for nuclear matrix isolation used in the various hybridization experiments are generally similar in approach, there are many differences in specific steps and their sequence. It has recently become apparent that minor changes in isolation conditions can result in major alterations in the morphology and composition of the isolated matrix fractions. For example, RNase treatment before high salt extractions (but not vice versa) results in matrices depleted of their characteristic internal structure (Adolph, 1980; Kaufmann et al., 1981). Also, insufficient cleavage of the nuclear DNA with nucleases leads to gel formation during high salt extraction which, in turn, greatly hinders isolation of nuclear matrix structures (Berezney & Coffey, 1975, 1977).

Differences in various matrix preparations might be more subtle but no less important. For instance, a number of studies have demonstrated that nuclear matrices are highly enriched with newly replicated DNA in vivo (Berezney & Coffey, 1975, 1976; Berezney, 1979b; McCready et al., 1980; Pardoll et al., 1980; Berezney & Buchholtz, 1981a). However, digestion of regenerating rat liver nuclei with DNAse I or micrococcal nuclease compared to endogenous digestion results in a preferential loss of replicating DNA fragments attached to the matrix (Berezney & Buchholtz, 1981a). Clearly, the method used for obtaining the nuclear matrix can profoundly influence the population of matrix-attached DNA fragments and result in selective removal or retention of specific sequences.

The organization of eukaryotic DNA into higher ordered loops may, aside from an obvious role for the three-dimensional arrangement and packaging of chromatin, also order the DNA into distinct functional units. For example, in the dynamic loop model which we propose, a rapid exchange of DNA with a fixed number of sites, while leading to a general random

association of DNA with the attachment sites, could also provide the physical basis for the regulation of specific loop formations during DNA replication and transcription. With regard to eukaryotic replication, it is interesting to note that the average mammalian chromosome contains thousands of replicon subunits which replicate in a seemingly nonuniform fashion along the DNA molecule (Edenberg & Huberman, 1975; Hand, 1978). However, now there is growing awareness that replicon synthesis is not a random process. Instead, clusters of replicons are regulated both spatially and temporally during replication (Hand, 1978). At the same time, evidence which suggests that the replicon units correspond to matrix attached DNA loops (Dijkwel et al., 1979; Pardoll et al., 1980; Berezney & Buchholtz, 1981a) is increasing, and a number of models have recently appeared which stress the dynamic nature of the DNA loops during replication (Dijkwel et al., 1979; McCready et al., 1980; Pardoll et al., 1980; Berezney, 1981; Berezney & Buchholtz, 1981a; Berezney et al., 1981b).

The possibile relationship of DNA loops to transcriptional units has also been extensively discussed (Georgiev et al., 1978). While the only definitive evidence for the organization of transcriptional units into distinct loops is derived from the specialized lampbrush chromosome systems (Scheer et al., 1979), Cook & Brazell (1980) concluded that transcriptional sequences are arranged in a specific manner along the DNA loops in HeLa cells, and Pardoll & Vogelstein (1980) reported an enrichment in ribosomal RNA sequences in matrix-attached DNA. Our hybridization analysis indicates that transcribed sequences are randomly associated with attachment sites, though it is still an open question as to whether transcriptional units are arranged into specific orientations along the DNA loops during the actual process of transcription.

Added in Proof

With regard to our proposal that the orientation of specific DNA loops at the matrix attachment sites may be a regulated phenomenon critical for nuclear function, Nelkin et al. (1980) reported that SV-40 DNA sequences are enriched at the matrix attachment sites of SV-40 transformed 3T3 cells.

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Diffusion-Driven Mechanisms of Protein Translocation on Nucleic Acids. 1. Models and Theory†

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ABSTRACT: Genome regulatory proteins (e.g., repressors or polymerases) that function by binding to specific chromosomal target base pair sequences (e.g., operators or promoters) can appear to arrive at their targets at faster than diffusion-controlled rates. These proteins also exhibit appreciable affinity for nonspecific DNA, and thus this apparently facilitated binding rate must be interpreted in terms of a two-step binding mechanism. The first step involves free diffusion to any nonspecific binding site on the DNA, and the second step comprises a series of protein translocation events that are also driven by thermal fluctuations. Because of nonspecific binding, the search process in the second step is of reduced dimensionality (or volume); this results in an accelerated apparent rate of target location. In this paper we define four types of processes that may be involved in these protein translocation events between DNA sites. These are (i) "macroscopic" dissociation–reassociation processes within the domain of the DNA molecule, (ii) "microscopic" dissociation–reassociation events between closely spaced sites in the DNA molecule, (iii) "intersegment transfer" (via "ring-closure") processes between different segments of the DNA molecule, and (iv) "sliding" along the DNA molecule. We present mathematical and physical descriptions of each of these processes, and the consequences of each for the overall rate of target location are worked out as a function of both the nonspecific binding affinity between protein and DNA and the length of the DNA molecule containing the target sequence. The theory is developed in terms of the *Escherichia coli lac* repressor–operator interaction since data for testing these approaches are available for this system [Barkley, M. (1981) *Biochemistry 20*, 3833; Winter, R. B., & von Hippel, P. H. (1981) *Biochemistry* (second paper of three in this issue); Winter, R. B., Berg, O. G., & von Hippel, P. H. (1981) *Biochemistry* (third paper of three in this issue)]. However, we emphasize that this approach is general for the analysis of mechanisms of biological target location involving facilitated transfer processes via nonspecific binding to the general system of which the target forms a small part.

1. Introduction

It is clear that in discharging many of their physiological functions (e.g., the processes of replication, transcription, translation, recombination, and repair) the proteins or protein complexes involved in various aspects of regulation of genome expression must translocate (move) along DNA or RNA molecules. Such translocation is generally unidirectional, proceeds at fairly well-defined rates, and requires the conversion of chemical to mechanical energy [for a recent summary, see Kornberg (1980)].

Simple protein–nucleic acid binding interactions are of two general types, and each may also involve various protein translocation mechanisms, though these are driven by diffusion processes (i.e., thermal fluctuations) only. These interactions include (i) the binding of regulatory proteins to one or a few specific target sites on the DNA genome (for example, the binding of repressors to specific operator sequences and the

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