replication. At the moment I favour the idea of an initiation factor which would be present in fraction IV of the Kornberg polymerase preparation. We can obtain routinely 70-fold replication of $d(TG)_n \cdot d(CA)_n$ and other synthetic polymers in the presence of fraction IV and pure polymerase. It seems unlikely that this is entirely the result of endonucleolytic nicks, especially because such nicks do not give rise to net-fold synthesis[14]. The other alternative, that fraction IV greatly increases the slippage rate, is not appealing.

I thank former colleagues at the Enzyme Institute, University of Wisconsin, for discussion and in particular the support of Dr H. G. Khorana. Dr V. H. Paetkau donated DNA polymerase, Drs H. van de Sande and M. Caruthers provided pyrophosphate diester model compounds and Dr M. Pearson the λ DNA. Dr R. D. Wells helped in the construction of CPK models. Miss Gayle Rutherford has repeated several experiments and Miss Terry Hukalo provided technical assistance. The manuscript was criticized by my colleagues at the University of Alberta. This work was supported by the Medical Research Council of Canada.

Note added in proof. Recently, experiments designed to label DNA using β,γ-^{32}P-labelled dNTPs as substrates yielded ^{32}P-labelled material that was excluded off agarose (15 M), was insoluble in TCA and resistant to alkaline, but did not band with DNA in a CsCl gradient. This throws some of the above interpretations into question. Although these results make a pyrophosphate link less likely, however, the basic principle of the model, namely, concomitant synthesis off both strands of DNA with daughter strands covalently linked, is still attractive, for modifications of the above model are readily constructed.

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T4 Bacteriophage Gene 32: A Structural Protein in the Replication and Recombination of DNA

by

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A new type of protein essential for DNA replication and genetic recombination has been isolated from T4 bacteriophage-infected cells of *E. coli*. This protein binds cooperatively to single-stranded DNA, and it catalyses DNA denaturation and renaturation in physiological conditions *in vitro*.

GENETIC recombination involves the precise breakage and reunion of "mating" double-stranded DNA molecules at points of mutual sequence homology[1–3]. Recombinant DNA molecules have been shown to contain a heterozygous region, which seems to be formed during the fundamental event in the recombination process[2,4]. Although the actual mechanism of genetic recombination is unknown, several relatively simple models have been proposed[5–7]; these assume an unusual fluidity of DNA structure within the cell which allows efficient testing for complementary base pairings between strands of randomly colliding DNA molecules. For example, in the scheme proposed by Holliday[5], local DNA denaturation is invoked to open mating DNA helices at homologous regions, followed by DNA renaturation between single strands thereby exposed on opposite molecules. *In vitro*, however, the DNA double-helix is overwhelmingly stable relative to the single strands in physiological conditions[8], and locally denatured regions more than a few base-pairs long should consequently occur only very rarely. This expectation is borne out by experimental studies of the stability of the short helix formed by the "cohesive ends" of isolated bacteriophage lambda DNA, for which, even in low [Na+] (0·033 M), transient melting of twelve contiguous base-

pairs occurs with a relaxation time of about 7 days at 37° C (ref. 9). By contrast, at 37° C within the cell, the average T4 DNA molecule participates in more than one recombination exchange every 10 min[10], whereas it must unwind completely during each round of DNA replication.

Single-stranded regions of DNA postulated as intermediates in various models for the recombination process could easily be generated by the action of exonuclease[7,11,12] or DNA polymerase[6] within the cell, if not by denaturation. It might be expected that the complementary single-stranded regions so generated would rapidly pair by renaturation because of the overwhelming stability of the double-helical conformation at 37° C. Single-stranded DNA folds on itself, however, to create imperfectly hydrogen-bonded, intra-strand helices in physiological conditions in vitro[13]. These folds make the DNA bases relatively inaccessible, and thereby prevent complementary single strands from finding satisfactory pairings[14,15]. As a result, raising the temperature from 37° C to 68° C increases renaturation rates as much as 1,000-fold. This is purely a kinetic effect, for the equilibrium stability of the double-helix relative to single strands is greater at the lower temperature.

We have discovered a DNA-binding protein in the T4 bacteriophage system the properties of which suggest a solution to this problem of DNA mechanics. The protein is the product of T4 gene 32. The "32-protein" is required for the genetic recombination of T4 bacteriophage DNA[16]; in addition, it is one of several gene products known to be essential for T4 DNA replication[17].

Biological Role of T4 Gene 32

The product of T4 gene 32 is required for T4 DNA replication throughout the infectious cycle: an amber mutant in this gene requires 40 min at 37° C to approximate even a single round of replication[18], whereas temperature-sensitive mutants which are allowed to begin synthesizing DNA at 25° C stop replication when shifted to a non-permissive temperature[19,20]. (For one particular mutant, ts P7, all replication ceases within 1 min after a shift to 42° C: S. Riva, A. Cascino, and E. P. Geiduschek, manuscript submitted for publication; unpublished results of M. Curtis and B. M. A.) Moreover, gene dosage experiments show that gene 32 is unique among the T4 genes known to affect DNA metabolism in that its product is required stoichiometrically rather than catalytically; that is, as for structural proteins of the phage particle, the quantity of 32-protein synthesized in the infected cell directly limits the number of progeny phage produced[21]. This finding must be reconciled with the fact that about 10,000 molecules of 32-protein are made in a normal infection, and very few, if any, are used up in the construction of mature phage particles[20]. It therefore seems that 32-protein plays a structural part in the replication of T4 DNA.

Another important biological observation concerning gene 32 is that, as first shown by Tomizawa and co-workers, its function is necessary for the formation of the hydrogen-bonded joint DNA molecules believed to be the initial products of genetic recombination[2,16,18]. In this connexion, it should be noted that DNA replication does not seem to be required for recombination of T4 DNA[16], and that recombination-deficient mutants in another bacteriophage system (phage λ) replicate their DNA normally[11]. It is therefore likely that 32-protein functions directly in both of these genetic processes.

Properties of Purified 32-Protein and its Binding to DNA

We have previously reported that at least twenty different DNA-binding proteins are synthesized after T4 bacteriophage infection of E. coli, as judged by DNA–cellulose chromatography[20]. One of the principal DNA-binding proteins was identified as the product of T4 gene 32, for it is altered after infection with bacteriophages carrying amber and temperature-sensitive mutations in this gene[19,20]. The 32-protein is made in large quantities at both early and late times of infection, about 10,000 molecules accumulating per infected cell.

In the absence of a direct assay for 32-protein, the course of its purification was originally monitored by polyacrylamide gel electrophoresis. In the work to be described stepwise elution from a single-stranded DNA–cellulose column followed by DEAE-cellulose chromatography has been used to prepare 32-protein which is electrophoretically homogeneous.

In spite of its tight binding to polyanionic DNA, 32-protein carries a net negative charge at pH 7. As estimated from a combination of sedimentation and gel filtration data, the molecular weight of the native protein is 35,000, and the axial ratio for an equivalent prolate ellipsoid is about 4 (ref. 19). Because the same molecular weight is obtained for denatured, reduced 32-protein in sodium dodecyl sulphate (SDS)-containing polyacrylamide gels[22], the native protein seems to consist of a single polypeptide chain[20].

Purified 32-protein binds strongly to single-stranded DNA, as seen by the co-sedimentation of ^{3}H-leucine-labelled protein with such DNA through stabilizing sucrose gradients. The affinity of 32-protein for DNA decreases gradually as the salt concentration is increased from 0·15 to 0·60 M, suggesting the importance of electrostatic forces in the binding. It seems likely, therefore, that the region of polypeptide chain in direct contact with the DNA includes a concentration of positively charged residues spaced so as to interact with the DNA phosphates, even though the protein as a whole carries a net negative charge.

The stoichiometry of the tight complex which 32-protein forms with single-stranded DNA at low salt concentrations has been examined by sucrose gradient sedimentation of a fixed quantity of labelled 32-protein in the presence of varying amounts of the circular, single-stranded DNA from bacteriophage fd[23]. At lower concentrations of DNA, two distinct peaks of radioactive protein are seen; one sediments rapidly with the DNA, the second at the slow rate characteristic of the free protein. The free protein peak is absent above a weight ratio of DNA to protein of 1 : 12. The complex therefore contains about one protein molecule of 35,000 molecular weight for every ten single-stranded DNA nucleotides. Because ten nucleotides can span a distance of not more than 70 Å, whereas 32-protein may be as much as 120 Å long, adjacent molecules of 32-protein could overlap in the complex. Consistent with this expectation, it was shown previously that in crude extracts 32-protein binds cooperatively to single-stranded DNA-cellulose[19].

To determine whether the purified 32-protein also binds cooperatively to DNA, two different concentrations of 32-protein (containing the same amount of tritiated 32-protein) were mixed with a constant amount (large excess) of fd DNA at an elevated salt concentration where the complex is only marginally stable. The results of sucrose gradient sedimentation analyses are shown in Fig. 1. It is clear that a 14-fold increase in 32-protein concentration dramatically increases its DNA affinity. This result requires that 32-protein molecules interact with each other in the complex (see caption to Fig. 1), and suggests a model in which there are two types of binding sites for 32-protein on single-stranded DNA: ten nucleotides adjacent to a previously bound molecule of 32-protein ("contiguous" site) and ten not adjacent to any previously bound molecule ("isolated site"). If this model is correct, the affinity of 32-protein for a contiguous site must be at least eighty times greater than its affinity for an isolated site, for strong cooperative binding is observed even in conditions where the number of isolated

sites available exceeds the number of contiguous sites by at least this factor (Fig. 1).

The highly cooperative nature of the DNA affinity of 32-protein should cause it to bind to DNA in long clusters even in conditions of large DNA excess. Direct evidence for such clustered binding is obtained when labelled 32-protein is mixed with a large excess of fd DNA and sedimented through sucrose gradients at low salt concentrations. In this experiment, a larger portion of the 32-protein sediments ahead of the main DNA peak, being tightly bound to a small fraction of the DNA molecules. The mean size of a 32-protein cluster in these conditions must therefore be an appreciable fraction of the length of an fd DNA molecule (6,600 nucleotides)[23]. Clustered binding to poly dA can also be detected by this method, indicating that the cooperativity observed is the result of direct stabilizing interactions between adjacent 32-protein monomers. This view is also supported by our finding (unpublished) that 32-protein self-aggregates in the absence of DNA at a concentration of 0·5 mg/ml. or higher.
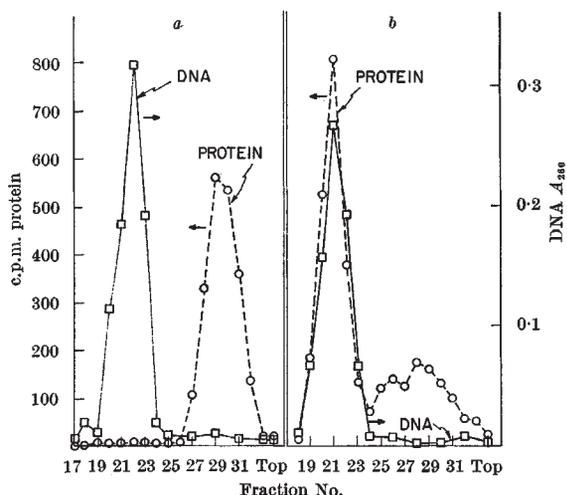

Fig. 1. Cooperative binding of 32-protein to single-stranded DNA. Purified fd DNA (10 μg) was mixed with about 0·5 μg (a) or 7 μg (b) of ³H-labelled 32-protein in 0·2 ml. of 0·02 M Tris-HCl (pH 8·1)—0·5 mM Na₂EDTA—0·30 M NaCl—100 μg/ml. bovine serum albumin (BSA)—10 per cent glycerol—1 mM β-mercaptoethanol at 4° C. After 20 min, the mixture was layered at 4° C onto a 5 ml., 5–30 per cent sucrose gradient prepared in the same buffer. Following centrifugation for 2 h at 46,000 r.p.m. in the Spinco 'SW50' rotor, 0·15 ml. fractions were collected and monitored for radioactivity by standard techniques. Recoveries of ³H-protein added averaged about 75 per cent. Concentrations of 32-protein (A₂₈₀=1·1/mg/ml.) and fd DNA (A₂₆₀=23·8/mg/ml. in 0·15 M NaCl—0·015 M sodium citrate, pH 7)²⁴ were determined by absorbance measurements. Note that, for ordinary binding, the protein distribution would have been identical in the above two experiments, for (free protein)/(bound protein)=K/(free DNA sites), and the concentration of free DNA sites was held essentially constant.

Although 32-protein binds very tightly to all single-stranded DNAs tested, including the synthetic polynucleotide poly dA, no binding of the purified protein to double-stranded DNAs or to R17 RNA could be detected by sucrose gradient sedimentation at 4° C.

Denaturation of DNA with 32-Protein

Histones and polyamines bind to the double-helical form of DNA more tightly than to single strands and thereby raise the temperature required for DNA denaturation[25,26]. Conversely, the strong selective affinity of 32-protein for single-stranded DNA should lower the thermal denaturation temperature of double-stranded DNA. A precedent for such an effect is the destabilization of DNA observed in the presence of pancreatic ribonuclease which likewise preferentially binds to DNA single strands[27].

Because single-stranded DNA is fully hyperchromic when complexed with 32-protein, any denaturation of

double-stranded DNA which occurs in its presence should be accompanied by the large hyperchromic change that is characteristic of this helix–coil transition. By this criterion, double-stranded T4 DNA is not denatured in the presence of excess 32-protein in a variety of ionic conditions at temperatures up to 37° C. By contrast, poly dAT, which normally has a T_m about 16° C lower than T4 DNA (65° C as against 81° C in 0·01 M KCl–0·01 M MgSO₄), is readily denatured by 32-protein even at 25° C. Typical kinetics for poly dAT denaturation by 32-protein are shown in Fig. 2. In the presence of 0·01 M Mg^{2+}, half-denaturation of poly dAT by 32-protein is attained in about 20 min in the conditions used; this denaturation is reversible, for the absorbance at 260 nm can be restored to its original value either by addition of NaCl to 0·5 M at 25° C (to dissociate 32-protein), or by direct cooling to 4° C. The initial rate of denaturation at 25° C is reduced with increasing $[Mg^{2+}]$, decreasing at least 15-fold when $[Mg^{2+}]$ is increased from 0·01 M to 0·04 M, and increasing about three-fold when all Mg^{2+} is removed.

To denature poly dAT at 25° C, ΔG for the coil→complex reaction (single-stranded DNA coil + 32-protein→ DNA – protein complex) must be sufficiently negative to make $\Delta G_{helix→coil} + \Delta G_{coil→complex} < 0$ at that temperature. The $\Delta G_{helix→coil}$ should be about $+1·0$ kcalories/ mole base-pairs for poly dAT in 0·01 M Mg^{2+} at 25° C ($T_m = 65°$ C) (ref. 28). Consequently, to obtain denaturation, $\Delta G_{coil→complex}$ will need to be < -5 kcalories/mole of binding sites (ten single-stranded DNA nucleotides) because $K_{dissociation} = \exp\ (-\Delta G/RT)\ \frac{(free\ protein)\ (free\ sites)}{(bound\ protein)}$,

half-denaturation of poly dAT in 0·01 Mg^{2+} at 25° C with 170 μg/ml. of 32-protein (Fig. 2) will require an effective dissociation constant for 32-protein of $< 1·1 \times 10^{-9}$ M. This is in agreement with direct measurements in sucrose gradients in similar conditions, which yield a dissociation constant (averaged for cooperactivity) of $< 10^{-9}$ M for the 32-protein complex with fd DNA.
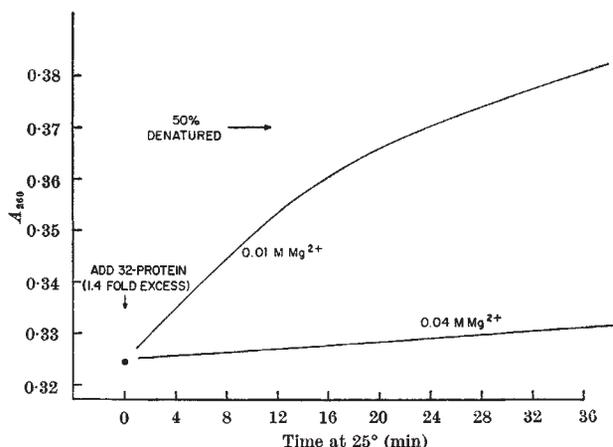

Fig. 2. Poly dAT denaturation catalysed by 32-protein at 25° C. In addition to the concentration of MgSO₄ indicated, each sample contained 10 μg/ml. of poly dAT (from A. Kornberg, s₂₀,w =29 in 0·1 M NaOH— 0·9 M NaCl), 170 μg/ml. 32-protein, 0·01 M KCl, 2 mM Tris-HCl (pH 8·1), 1 mM β-mercaptoethanol, 0·1 mM Na₂EDTA, and 2 per cent glycerol. Samples were placed in cuvettes in the thermostated compartment of a Gilford spectrophotometer, so that the absorbance could be monitored automatically. At time zero, concentrated 32-protein was added to start the reaction. Similar results have been obtained at a KCl concentration of 0·12 M.

Renaturation of DNA with 32-Protein

As already noted, the renaturation of purified DNA in physiological conditions in vitro is an extremely slow process, because of the intrastrand folding in denatured DNA. Our results imply, however, that in the T4 system single-stranded DNA does not exist as such in vivo, but is instead always present as a tight complex with 32-

protein. We find that fd DNA saturated with 32-protein sediments only about 1·3 times faster than the free DNA, although its mass is thirteen times greater. This means that the frictional coefficient of fd DNA increases about six-fold in the complex. Because frictional coefficients only double when single-stranded DNA is unfolded in alkali[29], complexed DNA must be held in a highly expanded conformation by 32-protein. (This expansion can be seen directly by electron microscopy; personal communication of H. Delius.) DNA in such a conformation might be expected to renature much more rapidly than free denatured DNA at low temperatures.

The rate of renaturation of DNA covered with 32-protein was measured by an absorbance assay similar to
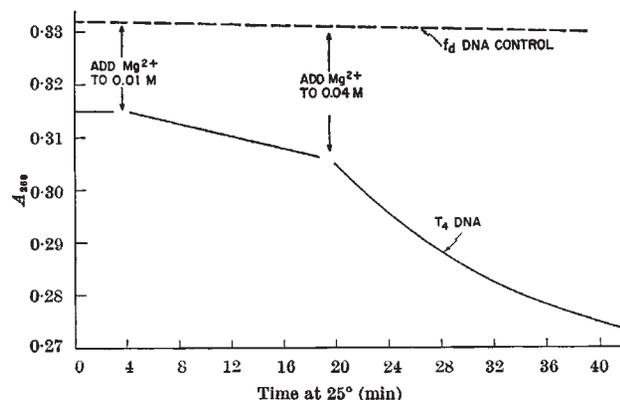

Fig. 3. DNA renaturation catalysed by 32-protein at 25° C. Single-stranded T4 DNA was prepared by alkaline denaturation and dialysis into low salt buffer as described by Studier[15], except that the DNA was sheared in alkali to molecular weight 5·4 × 10⁶ before the dialysis. The fd DNA serves as a control, for it is not self-complementary and therefore cannot renature. At zero time, 10 μg/ml. of each DNA was mixed in separate cuvettes with 170 μg/ml. of 32-protein in the buffer used in Fig. 2. At the times indicated, MgSO₄ was added from a 1 M stock to both fd and T4 DNA reactions. A decrease in absorbance of about 0·072 units is expected for full renaturation of the T4 DNA.

that used to monitor denaturation in Fig. 2. In this case, a decrease in the absorbance of single-stranded DNA is expected proportional to the amount of reformed double-helix. Typical results obtained for single-stranded T4 DNA in the presence of excess 32-protein at 25° C are presented in Fig. 3. It can be seen that a rapid decrease in absorbance is observed in 0·04 M Mg²⁺, representing a more than 1,000-fold acceleration of the renaturation rate without 32-protein. The dependence of this reaction on [Mg²] is the reverse of that found for denaturation in Fig. 2: the renaturation rate drops about four-fold in 0·01 M Mg²⁺, whereas no renaturation is detected without Mg²⁺.

A more sensitive measure of the course of renaturation is obtained from CsCl gradients, where renatured DNA has a lower buoyant density than single strands[14]. This assay

Table 1. RENATURATION RATES FOR T₄ DNA SINGLE STRANDS OF MOLECULAR WEIGHT 5·4 × 10⁶

32-Protein	DNA concentration (μg/ml.)	Ionic composition	Temperature	(K_2 l. mole⁻¹ s⁻¹)
—	15	1·0 M NaCl	68°	270
—	76	0·04 M MgSO₄ 0·01 M KCl	37°	< 0·2
+	15	0·04 M MgSO₄ 0·01 M KCl	37°	300
+	72	0·011 M MgSO₄ 0·12 M KCl	37°	22

For the 32-protein catalysed reaction: (1) renaturation rates decrease with storage of the protein, so that the maximum rates are probably greater than those listed; (2) between 0·01 M and 0·04 M Mg²⁺, the rate at 37° C is roughly proportional to [Mg²⁺]; it falls drastically at lower Mg²⁺ levels; (3) renaturation rates are reduced as [KCl] is increased, being severely affected above 0·15 M; and (4) addition of spermidine (0·001 M) is without significant effect. The rates shown were measured by CsCl banding after incubation at pH 7·6 (the catalysed reaction has a broad optimum between pH 7 and pH 8). Enough 32-protein was used fully to saturate the DNA.

can be used for kinetic analyses, for the addition of concentrated CsCl dissociates 32-protein from the DNA and prevents further renaturation. By this technique, it has been found that the rate of renaturation of T4 DNA in the presence of excess 32-protein is proportional to the square of the DNA concentration, showing that, as in the uncatalysed reaction, the rate measured is that for the nucleation of complementary pairings. Some second order rate constants for the renaturation of T4 DNA single strands of molecular weight 5·4 × 10⁶ are listed in Table 1. Note that the rate of renaturation catalysed by 32-protein at 37° C can exceed the uncatalysed rate observed in standard conditions (68° C in 1 M NaCl).

If 32-protein accelerates DNA renaturation by imparting favourable conformation to the single strands, the bases of which would otherwise be inaccessible on highly folded chains, the dependence of renaturation rate on the ratio of 32-protein to DNA should be unusual. Below the saturating protein : DNA ratio of 12 : 1, the rate of renaturation should be drastically lowered, initial rates being proportional to at least the square of the amount of 32-protein added. Results of renaturation assays performed at sub-saturating 32-protein levels are shown in Fig. 4, where it is seen that a four-fold drop in the concentration of 32-protein decreases the rate of renaturation of T4 DNA at least twenty-five-fold, as expected. A second expectation is that above a protein : DNA ratio of 12 : 1, additional 32-protein should not further increase renaturation rates. This prediction has also been confirmed (experiment not shown).

On the basis of these results, we conclude that 32-protein accelerates renaturation in physiological conditions by forcing DNA single strands into an unfolded conformation which leaves their bases available for pairing during chance collisions between complementary strands. It seems likely that the bound 32-protein is rapidly displaced from the rewinding single strands as the double-helix forms (see Fig. 3).
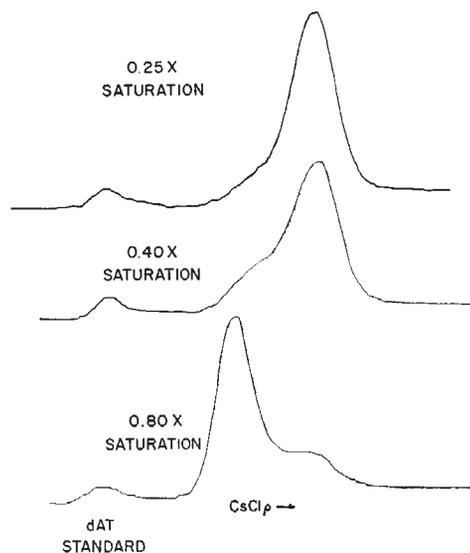

Fig. 4. T4 DNA renaturation as a function of 32-protein : DNA ratio. Renaturation was carried out with T4 DNA single strands (Fig. 3) at 72 μg/ml., and the quantity of 32-protein added was varied as indicated. Incubation was at 37° C in buffer containing 0·12 M KCl and 0·011 M MgCl₂. After 30 min, the reaction was quenched by addition of concentrated CsCl (to ρ = 1·700) plus 50 μg of sodium dodecyl sulphate. The band patterns shown are tracings of photographs taken after centrifugation for 20 h at 44,770 r.p.m. in the Spinco model E analytical ultracentrifuge. The renatured band has shifted to a buoyant density representing molecules which are about two-thirds double-helical, as expected from the pairing of strands randomly cut from a longer sequence[30]. As controls, no density shift was observed when fd DNA was processed in an identical manner, whereas fragments of single-stranded T7 DNA renatured rapidly in the presence of 32-protein.

Function of 32-Protein in Genetic Recombination

The denaturation of poly dAT by 32-protein at 25° C indicates that within the cell, the cooperative action of 32-protein should generate fluctuating regions of local denaturation in T4 DNA. The requirement for 32-protein in early steps of genetic recombination can be explained by this ability to open up local regions of native DNA, while simultaneously facilitating helix formation between matching, complexed single strands. Both of these functions are probably necessary for efficient testing of complementary pairings between double-stranded DNA molecules. In addition, experiments with infected cells have suggested that the formation of single-strand breaks ("nicks") in double-stranded intracellular T4 DNA is a prerequisite for the initiation of strand exchanges[31]. Order-of-magnitude calculations, based on the treatment of DNA renaturation formulated by Wetmur and Davidson[30], suggest that random collisions between specifically nicked, double-stranded T4 DNA molecules (see ref. 5) might be efficient enough in the presence of 32-protein to account for the high recombination rates observed in the T4 system (unpublished results). This, however, merely indicates that the mechanism of genetic recombination may be relatively simple, for the properties of 32-protein are also compatible with most other types of models suggested for the recombination process.

Function of 32-Protein in DNA Replication

The genetic results which indicate a structural role for 32-protein in DNA replication suggest that local unwinding by 32-protein might be required in the replication fork in order for productive replication to proceed. In agreement with this role, in vitro experiments have revealed that T4 DNA polymerase[32] uses single-stranded DNA templates much more rapidly in the presence of 32-protein than in its absence (unpublished results of Huberman, Kornberg and B. M. A.). This stimulation is probably the result of favourable template alignment by 32-protein. Because stimulation is not observed in similar experiments with E. coli DNA polymerase, a direct interaction of T4 polymerase with 32-protein may also be involved.

In a normal T4 bacteriophage infection, the number of replication forks present per cell increases linearly until 30 min after infection (25° C)[33]. Because the rate of polymerization observed at each fork is constant throughout this period[33], this rate must be independent of the level of 32-protein, inasmuch as this increases continuously during infection. Yet the gene dosage experiments, which reveal a direct proportionality between the quantity of phage progeny produced and the quantity of 32-protein present, seem to demand that the overall rate of DNA synthesis be proportional to the amount of 32-protein made[21]. To account for these facts, we propose that a functioning replication fork has a unique tertiary structure that contains a fixed number of 32-protein molecules. (About sixty new replication forks are eventually generated in a normal T4-infected cell, so that each fork could incorporate no more than 170 molecules of 32-protein.) In our view, the amount of 32-protein determines the quantity of DNA made, for a new replication fork can be formed only as fast as a threshold level of free 32-protein becomes available.

If each cycle of DNA replication begins at a special point on the T4 genome[34], new replication forks must be generated only at a unique nucleotide sequence. Both during this process and as the replication fork travels, 32-protein may interact with other proteins in addition to T4 DNA polymerase. Likely candidates for such proteins include the products of T4 genes 41, 44, 45, 59 and 62, all of which have as yet unidentified functions essential for T4 DNA replication[17]. Further studies involving 32-protein may therefore provide a fresh insight concerning the unknown mechanism by which DNA is replicated in biological systems.

Preparation of Homogeneous 32-Protein

An E. coli culture, grown to 5×10^8 cells per ml. at 32° C in M-9 minimal media containing 0·3 per cent casein hydrolysate plus 1 per cent glucose, was infected twice at 10 min intervals with a total m.o.i. of ten T4e bacteriophages (lysozyme⁻). The cells were harvested and washed after 90 min of aeration at 32° C and stored at −20° C. Cells (50 g) were broken by sonication after resuspension in 200 ml. of 0·02 M Tris-HCl (pH 8·1)—0·01 M $MgCl_2$—2 mM $CaCl_2$—1 mM β-mercaptoethanol—1 mM Na_3EDTA containing 20 μg/ml. pancreatic deoxyribonuclease I (Worthington). After incubation for 90 min at 10° C, the extract was centrifuged at low speed to remove cell debris and then clarified at 30,000 r.p.m. for 3 h in the Spinco 30 rotor. The supernatant was dialysed for 24 h against several changes of 0·02 M Tris-HCl (pH 8·1)—0·05 M NaCl—5 mM Na_3EDTA—1 mM β-mercaptoethanol (buffer A) to remove the divalent cations necessary for the activity of deoxyribonuclease I. After centrifugation to remove a light precipitate, the dialysed extract was made 10 per cent in glycerol and forced at 100 ml./h through a column containing 20 ml. packed volume of denatured calf thymus DNA–cellulose (approximately 1 mg of DNA per ml.). The DNA–cellulose (7 cm × 3 cm²) had been equilibrated with a buffer consisting of 10 per cent glycerol in buffer A, and this basic buffer was used for an 80 ml. rinse and for elutions in which increasing concentrations of NaCl were added. The column was eluted at 20 ml./h, in 40 ml. steps of 0·15, 0·40, 0·60 and 2·0 M NaCl. The peak 2·0 M NaCl eluting fraction contained 32-protein as its principal component. This fraction (8 ml.) was dialysed against 0·02 M Tris-HCl (pH 8·1)—10 per cent glycerol—1 mM Na_3EDTA—1 mM β-mercaptoethanol (buffer B) and applied to a 7 cm × 0·8 cm² column of DEAE–cellulose (Whatman DE32). The column was washed with 5 ml. of buffer B and then eluted with a 30 ml. linear gradient of 0–0·5 M NaCl in this buffer. Fractions of 1·3 ml. were collected every 20 min. The 32-protein (A_{280}/A_{260} absorbance ratio of 1·7) appeared in three adjacent fractions with a mean NaCl concentration of 0·20 M. These fractions were either used directly for the studies to be described, or concentrated further by vacuum dialysis against buffer B containing 0·05 M KCl. Approximately 8 mg of electrophoretically homogeneous 32-protein is obtained by this procedure. As determined by the subsequent recovery of purified ³H-labelled 32-protein added to crude extracts, this represents about a 65 per cent yield. An identical procedure was used on a smaller scale for preparation of ³H-labelled 32-protein, except that the cells were grown at 25° C and labelled with 500 μCi of ³H-leucine after 35 min of infection. Unless otherwise stated, all operations were carried out at 4° C; at this temperature, concentrated solutions of 32-protein (> 500 μg/ml). may be kept for several weeks. At −80° C, 32-protein has been stored for up to 8 months without a noticeable change in its DNA affinity.

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Three Variable-Gene Pools common to IgM, IgG and IgA Immunoglobulins

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Comparison of the NH_2-terminal region of several α, γ and μ chains indicates that four variable-sequence subgroups are common to heavy chains. The existence of three independent variable-gene pools common to the three major immunoglobulin classes is confirmed.

IMMUNOGLOBULINS can be divided into three principal classes according to their different antigenic and functional properties. The class differences reside in the heavy chains, whereas the light chains, which are chemically and immunologically defined as $\varkappa$ or λ chains, are shared by all three immunoglobulin classes[1,2]. The IgA immunoglobulin class is characterized by the α chain, IgG by the γ chain and IgM by the μ chain. In spite of these structural and functional differences certain common features exist for the three chief classes. A common evolution for all immunoglobulins is evident from comparison of the chemical structure of heavy and light chains from different species[2,3]. Furthermore, there is a general cooperative and sequential relationship in the immune response of all three immunoglobulin classes. Finally, serological cross-reactions between IgM and IgG have been observed by a number of workers. In fact, the earliest evidence for a heavy chain gene was the finding by Todd[4] of a common allotypic marker in rabbit IgG and IgM. Thus the question arises where in the primary structure of the α, γ and μ chains the differences and similarities of the three principal immunoglobulin classes are localized. Earlier[5] we made the first comparison of an extended sequence of a μ chain (Ou) with a $\gamma1$ chain (Daw)[6] which showed that in the first 105 NH_2-terminal residues of these two human heavy chains of different classes the homology in sequence was almost 75 per cent. Subsequently, we undertook sequence analysis of several human IgM Waldenström macroglobulins and IgA myeloma proteins to ascertain if this high degree of homology of two different heavy chain classes was merely accidental. We now report additional heavy chain sequences from the NH_2-terminus of four IgM macroglobulins and one IgA myeloma globulin. Comparison of these data with the NH_2-terminal sequences of heavy chains from other laboratories has revealed homologies of different degrees which suggest the existence of two new variable heavy chain subgroups, V_{HIII} and V_{HIV} in addition to the V_{HI} and V_{HII} subgroups already proposed[7,8].

Determination of Amino-acid Sequence

Because most of the heavy chains in immunoglobulins have a blocked NH_2-terminal residue which is the cyclized form of glutamine (pyrrolidone carboxylic acid or PCA)[9,10], a specific method to isolate blocked NH_2-terminal peptides from the whole molecule can be used[11]. IgM protein (1–2 μmoles) was digested with subtilisin for 2 h at 37° C and pH 8. The soluble digest was then applied to a 'Dowex 50×2' column previously washed with 1 M HCl and distilled water until neutral. The first peak eluted contained glycopeptides and the second peak a tetrapeptide which was ninhydrin-negative on paper. The amino-acid composition of the tetrapeptide from proteins Ou, Di and Re varied in only one residue. In all three cases a 10 min and 120 min incubation with carboxypeptidase A released two residues in different yields. By this method the amino-acids at positions three and four were established. Because the first residue could be assumed to be PCA, the residue in position two could be deduced. In another experiment, we determined the partial sequence of the nineteen NH_2-terminal residues of protein Di by study of tryptic peptides from the NH_2-terminal fragment obtained by CNBr cleavage.

Attempts to isolate a blocked peptide failed with the IgM proteins Wo (personal communication from A. van Dalen) and Na and with the IgA protein Ha. But we found a free NH_2 terminus on these heavy chains by means of the dansylation technique[12]. The NH_2-terminal residue in the unblocked heavy chains of proteins Wo, Na and Ha was glutamic acid. These results confirm earlier data from this laboratory which showed that in about half of the μ chains studied the NH_2 terminus was not blocked[10]. The μ chains of IgM Wo and Na and the α chain of IgA Ha were subjected to the automatic Edman degradation method[13] using the Beckman sequencer model 890. Identification of the degraded PTH-amino-acid was done by thin-layer chromatography[14] and by gas chromatography using a combination of three different columns[15]. In one