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Synthesis of Block Oligonucleotides

Abstract. *The concentration of sodium chloride strongly influences primed polymerization of nucleoside diphosphates by polynucleotide phosphorylase. High concentrations of NaCl allow the addition of only a few nucleotide residues onto the 3′ end of the primer. When pure oligonucleotides are used as primers and when the product is fractionated, pure block oligonucleotides can be obtained.*

Highly purified preparations of polynucleotide phosphorylase from *Micrococcus lysodeikticus* show a nearly absolute requirement for oligonucleotide primer in the polymerization of nucleoside diphosphates (*1*). Singer, *et al.* (*2*) have shown that primers serve as chain initiators in the following manner:

$$\text{NDP} + \text{NDP} \longrightarrow \text{little or no reaction}$$

$$(\text{pN})_n + \text{NDP} \rightleftharpoons (\text{pN})_{n+1} + \text{P}_i$$

where NDP is nucleoside diphosphate, $(\text{pN})_n$ is polynucleotide, and P_i is inorganic phosphate. At saturating primer concentrations virtually every polynucleotide chain that is formed contains a primer residue at the 5′ end (*3*). Thus, in principle, this reaction should provide a simple method for the synthesis of short two-component block copolynucleotides. However, there are two major obstacles to the general application of this method which have until now restricted its practical use to the synthesis of very long homopolynucleotide chains containing two or three heterologous bases at the 5′ end of the chain (*4*).

The first obstacle is that the reaction is readily reversible. Thus the partial phosphorolysis of any of the primer oligonucleotides present in the reaction mixture must be prevented. The second difficulty arises from the fact that under normal conditions chain initiation is much slower than chain growth (*5*). As a result, the short, primed oligonucleotide intermediates which we seek are present only in very small amounts throughout the course of the polymerization (*3*, *6*). Both these problems can be eliminated simply by choosing the proper set of reaction conditions.

Polynucleotide phosphorylase from *M. lysodeikticus* was purified 90-fold (*1*). The fractions used in our experiments were virtually free of nuclease and contained negligible phosphatase activity. A nearly absolute requirement for primer was observed under normal reaction conditions, polymerization rates being increased 15 to 20 times by the addition of saturating amounts of primer (*7*, *8*). We have found that the scanty endogenous enzymatic activity which remains after extensive purification (*1*) is completely inhibited by high concentrations of NaCl (above 0.4*M*), a condition which prevails throughout all the polymerization reactions described below. This inhibition is observed not only at *p*H 8.1 but also at *p*H 9.3, and it is found even with relatively crude enzyme preparations which, in the absence of salt, are not stimulated by primer at the higher *p*H.

Oligocytidylic acid $[\text{C}(\text{pC})_5]$ was labeled with C^{14} and used for priming the polymerization of uridine diphosphate (UDP) at two different NaCl concentrations. The course of polymerization was followed by removing samples from the reaction mixture at intervals and spotting them on chromatography paper. The chromatograms were then developed in a solvent composed of 95 percent ethanol and 1*M* ammonium acetate (60 : 40 by volume). In this solvent unincorporated primer migrated away from the origin, while chains longer than eight residues were immobile (*9*). After the chromatograms were dried, origin spots were eluted, and the amount of polymer was determined spectrophotometrically; the amount of incorporated primer was determined by measurements of radioactivity. The ratio of these two parameters, (nucleotide residues) / (primer residues), gave the number-average degree of polymerization of the product.

The progress of the polymerization can now be followed in three ways (Fig. 1). The higher NaCl concentration (i) depresses the rate of UDP polymerization, (ii) leaves unaffected the complete utilization of primer, and (iii) yields a product of quite short chain length. Thus NaCl terminated chain growth at a very early stage so that primed oligonucleotides accumulated. This NaCl effect is not simply a general noncompetitive inhibition of the enzymatic active site; the rate of primer incorporation is, if anything, slightly increased, whereas the rate of chain growth is drastically reduced. Moreover, when the reaction is completed, only 5 percent of the nucleoside diphosphate originally present has reacted to

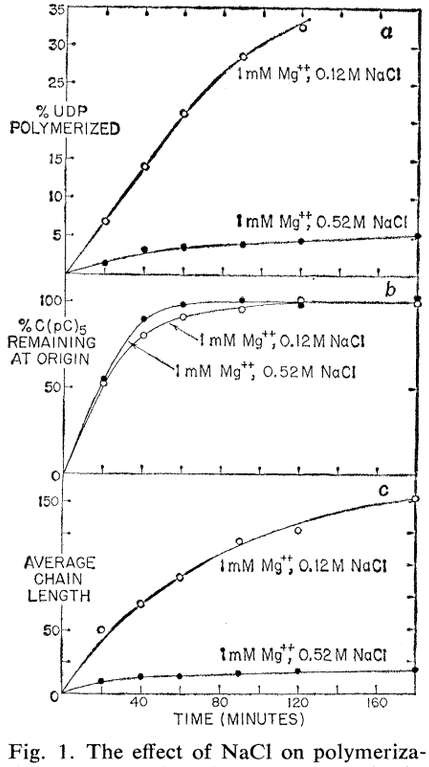

Fig. 1. The effect of NaCl on polymerization kinetics. The salt concentration in the reaction mixture was varied by the addition of NaCl. Incorporation of radioactive primer was determined by adding a portion of the eluates from the origin spots of the chromatogram to 20 ml of scintillator gel (Bray's solution (*12*), made 6 percent in Cab-O-Sil) and counting in a liquid-scintillation counter. The average chain length is calculated as the mole ratio of polymer residues to incorporated primer residues.

give polymer and P_i. Hence the phosphorolysis reaction, in which P_i is a reactant, would be negligible under these conditions. Indeed, analysis shows that none of the radioactive primer was phosphorylyzed to the corresponding diphosphate. Hence these reaction conditions are ideally suited for the synthesis of short block oligonucleotides.

The dependence of the chain length of the product on NaCl concentration is shown in Fig 2. The primers were $C(pC)_5$, $C(pC)_7$, and $C(pC)_8$. No relation between primer size and polymer chain length was observed under these conditions. However, at much lower salt concentrations (less than 0.15*M*) polymer chain length does depend strongly on primer size (*3*). Another interesting feature (Fig. 2) is that at low Mg^{++} concentration (1m*M*) the polymer chain length was independent of the type of nucleoside diphosphate polymerized. However, when the Mg^{++} concentration was increased ten times, oligoadenylic and oligocytidylic acid chains grow to about twice the length of oligouridylic acid chains.

The polymerization of inosine diphosphate (IDP) is unique in that two to three times more salt is required to inhibit chain growth than for any other nucleotide, regardless of Mg^{++} concentration. On the other hand, guanosine diphosphate (GDP) apparently does not behave very differently from adenosine diphosphate (ADP). As yet we have no explanation for this variation in behavior among the different nucleotides.

Other factors besides NaCl and Mg^{++} concentration may influence polymer chain length. Both temperature and the presence of urea affect the size of the polymer product. Thus at 37°C polyuridylic acid chains 18 to 22 units long are formed, whereas at 33°C the chain length increases to 30 to 35 residues (1m*M* Mg^{++}; 0.52*M* NaCl). Similarly, a reaction mixture, 1*M* in urea, produces polyadenylic acid chains only 40 percent as long as those synthesized in the absence of urea (10m*M* Mg^{++}; 0.7*M* NaCl).

Although we have generally used relatively short oligonucleotides (6 to 9 residues) as primers, much longer chains may be used with equal success. For example, we have encountered no difficulty in synthesizing the following block copolymers: $U_{\overline{14}}C_{\overline{6}}$, $U_{\overline{14}}A_{\overline{5}}$, $U_{\overline{17}}I_{\overline{3}}$, $I_{\overline{21}}U_{\overline{16}}$, $I_{\overline{21}}C_{\overline{5}}$ (in these cases the lengths in units of each component of the block are number-average chain lengths,

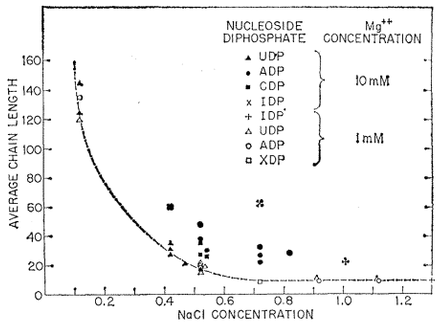

Fig. 2. The relation between sodium chloride concentration and average chain length. The Mg^{++} concentration in the reaction mixture was varied as indicated. Since the average length of the chain increases with time (Fig. 1), the values plotted correspond to the point at which 90 percent of the primer is incorporated into polymer. The broken line is drawn through all points obtained from reactions containing 1m*M* Mg^{++}, except for those in which IDP was a reactant.

as designated by a bar over the numerical subscript). On the other hand, we have met with only limited success in making polymers of the type $A_{\overline{15}}U_{\overline{15}}$ or $I_{\overline{20}}C_{\overline{20}}$. In these cases hydrogen bonding between the two components may be responsible for inhibiting the growth of the second component beyond 10 to 15 residues. This problem may presently be solved by finding conditions—such as high *p*H or the presence of urea (*10*)—which prevent hydrogen bonding yet allow polymerization to proceed.

As a result of these studies it is now possible to make block copolynucleotides of many sizes and varying properties. Moreover, the methods described can be used in the stepwise synthesis of an oligonucleotide containing internally any desired short sequence of bases. This may be done by conducting the polymerization reaction under conditions which allow the addition of only a few residues to the primer, after which the heterogeneous product is fractionated chromatographically. The desired species may then be isolated and used to prime a second polymerization with a different nucleoside diphosphate. The only limitation to the size of polymer that can be built up in this fashion is the resolving power of the chromatographic techniques. For example, we have synthesized the oligonucleotide $C_5A_1U_{\overline{25}}$ by using C_5 to prime the end-addition of ADP in the presence of a high NaCl concentration (1m*M* Mg^{++}, 1*M* NaCl), after which the reaction mixture was chromatographed in a solvent composed of 95 percent ethanol and 1*M* ammonium acetate (55:45 by volume). The oligonucleotides C_5A_1, C_5A_2, and C_5A_3 were then eluted and concentrated by lyophilization. The chain lengths of oligonucleotides were determined by chromatographic mobility. An independent check on this method was routinely obtained with labeled nucleoside diphosphate in the reaction mixture. Thus, the chain length of a given chromatographic component in the reaction product can be determined from absorbance and radioactivity measurements as described. Finally, the purified oligonucleotide C_5A_1 was used for priming the polymerization of UDP in the usual way (10m*M* Mg^{++}, 0.47*M* NaCl.)

The mechanism by which NaCl, as well as other agents, inhibits chain growth is not understood; however, it seems likely that the site of action is the enzyme molecule itself, rather than the oligonucleotide substrate, since the chain lengths of polynucleotides of very different physical properties are all affected in the same way by a particular agent. Block copolymers of the type described have proved very useful (*11*) in studying the mechanism and specificity of protein synthesis in vitro.

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