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Hexamine cobalt chloride promotes intermolecular ligation of blunt end DNA fragments by T4 DNA ligase

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ABSTRACT

Hexamine cobalt chloride (HCC) increases the efficiency of blunt end ligation by T4 DNA ligase about 50 fold. Maximum stimulation occurs when standard buffers for ligation are supplemented with 1 mM HCC. All the ligation events are intermolecular regardless of the initial DNA concentration. In the presence of monovalent cations (eg. 25 mM KCl) HCC still increases the extent of T4 catalyzed ligation but intramolecular ligation products are also formed. Therefore, intermolecular ligation can be performed rapidly and at low DNA concentrations.

INTRODUCTION

The joining of DNA fragments by DNA ligase (1,2) is a central step in strategies to clone DNA sequences. The most widely used ligase for this purpose is T4 DNA ligase because this enzyme can catalyze the linkage of blunt end DNA fragments (3,4). Joining of blunt end fragments is an inefficient reaction that requires high concentrations of DNA and enzyme for intermolecular ligation. Recent studies show volume exclusion (5) using high concentrations of polyethylene glycol or other polymers (6,7) can increase the rate of blunt end ligation. Also, a protein isolated from Xenopus ovaries can enhance intermolecular ligation (concatenation) to produce linear multimers (8).

While examining the ability of recA protein to aggregate duplex DNA thus enhancing intermolecular ligation (9) we found that hexamine cobalt chloride (HCC) dramatically increases the efficiency of T4 ligase catalyzed ligation. While HCC has previously been suggested for ligation of linker oligonucleotides (10) we found HCC most effective in enhancing ligation of larger DNA fragments. Experiments presented here demonstrate that T4 ligase catalyzed ligation in 1 mM HCC enhances blunt end ligation about 50 fold and all ligation events are intermolecular. We also show that when 30 mM KCl was

present ligation was enhanced by HCC but intramolecular ligation events predominate. We suggest that cation induced DNA condensation (11-14) effectively increases the local concentration of DNA ends thereby allowing joining of two DNA fragments even at low DNA concentration.

EXPERIMENTAL PROCEDURES

Preparation of DNA

Plasmid pACYC184 was maintained in E. coli strain AB2487 (recA13). Previously described methods were used to prepare plasmid DNA (15) and ϕX174am3 RF I DNA (16). Full length linear molecules were produced from form I DNA by restriction enzymes using conditions prescribed by the manufacturer. All DNA was dialyzed to equilibrium against 10 mM tris-HCl pH 7.5, 0.5 mM EDTA prior to use. Unless otherwise indicated, DNA concentrations are expressed as the molarity of DNA ends.

The 12 basepair fragment 5'dCCGGAATTCCGG (New England Biolabs) was end labeled using [γ-^{32}P]ATP and T4 polynucleotide kinase (P-L Biochemicals) by established procedures (17).

Ligation Reactions

Ligation of DNA termini can be either intermolecular or intramolecular and the relative amount of each product can be theoretically calculated (18). The probability of an intramolecular ligation event is expressed as the ratio i/J where J is the effective concentration of one end of a DNA molecule in relation to the other end and i is the total concentration of DNA ends. At a i/J ratio of 1 there is a 50% probability of joining two ends of the same molecule. As i increases there is a higher probability of intermolecular ligation events.

Typical ligase reactions were performed in a mixture (20 µl) containing 25 mM tris-acetate pH 7.5, 5 mM $MgCl_2$, 0.1 mM ATP, 0.5 mM dithiothreitol and the indicated amount of linear DNA. Hexamine cobalt chloride ($(NH_3)_6CoCl_3$; Aldrich Chemical Co.) was added to 1 mM where indicated. T4 DNA ligase from New England Biolabs was used in all ligation experiments. Samples were routinely incubated at 23°C for 10 min and the reaction stopped by the addition of EDTA to 50 mM and SDS to 0.5%. Ligation units are defined as that amount required to give 50% ligation of Lambda Hind III fragments in 30 min at 16°C when the DNA termini concentration is 0.12 µM or above. This

unit is equal to 0.015 pyrophosphate exchange units (19) or 0.0025 circle formation units (20).

Linear multimers formed by ligation in the presence of 1 mM HCC were purified by preparative gel electrophoresis. The standard reaction mixture was scaled up to 0.45 ml and contained 12.5 nM of ϕX174 [^{32}P]DNA linearized with Ava II and 20 units of T4 ligase. After 15 min at 23^{o}C the reaction was terminated as above and electrophoresed through a 0.9% agarose gel. The linear multimers were obtained by electroelution from an agarose gel strip placed in a dialysis bag. The sample was phenol extracted and ethanol precipitated. The purified multimers required higher than normal levels of restriction enzymes (5-15 units/μg DNA) to be completely digested. Ligation products restricted normally prior to preparative gel electophoresis.

RESULTS

Hexamine Cobalt Chloride Stimulates Ligation of DNA Fragments by T4 DNA Ligase

We examined the effect of $(NH_3)_6CoCl_3$ on ligation of DNA fragments by T4 DNA ligase. Blunt end linears of ϕX174 duplex DNA produced by digestion with Stu I were incubated at 24^{o} for 10 min with 1.2 units of T4 DNA ligase. Under these conditions few ligation events occur because of the low efficiency of ligating blunt end DNA fragments. Reaction products were observed by electrophoresis of the DNA through an agarose gel.

When reaction mixtures were supplemented with increasing concentrations of hexamine cobalt chloride a dramatic increase in the amount of ligated fragments was observed (fig 1). Maximum stimulation was observed at 1mM HCC although little or no stimulation was seen at 0.5 mM. This sharp transition was reproducible and consistent stimulation was observed at 1-1.5 mM hexamine cobalt chloride. $CoCl_2$, $CuCl_2$, $RbCl_2$, $ZnCl_2$, $MnCl_2$, and $NiCl_2$ were ineffective in stimulating ligation by T4 DNA ligase (data not shown).

Duplex DNA fragments containing complementary single strand ends are also ligated to a greater extent in the presence of 1 mM HCC. Titration of ligase in the presence or absence of HCC and electrophoretic separation of ligated products allows a comparison of the extent of ligation after 10 min (fig 2). At 1.8 nM DNA ends (i/J=0.1), ligation of DNA with four base 3' single strand ends was stimulated about 5 fold by HCC. Similar results were obtained with 5' single strand ends (data not shown). When blunt end DNA was used HCC

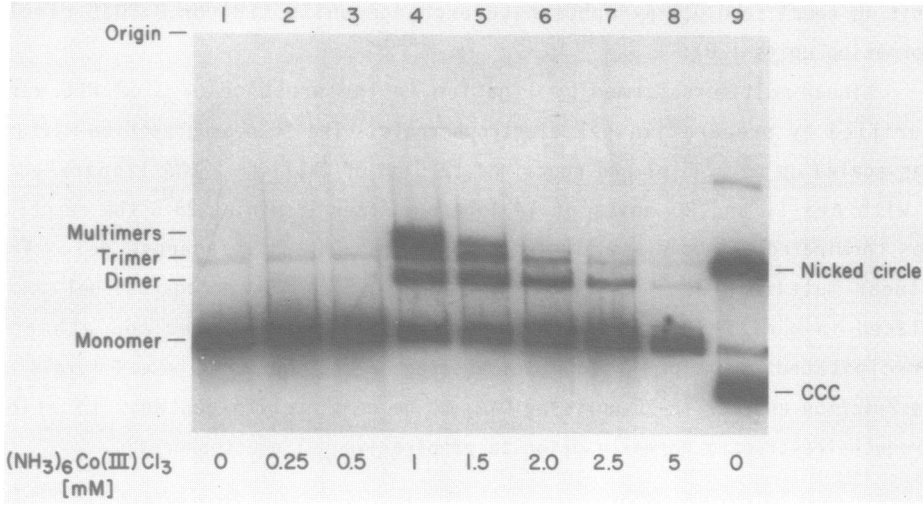

Fig 1. Blunt end ligation by T4 ligase in the presence of HCC.
A reaction mixture (20 µl) containing 25 mM tris-acetate, 5 mM $MgCl_2$, 0.1 mM ATP, 0.5 mM dithiothreitol, 3.7 nM blunt end ϕX174 [^{32}P]DNA generated by digestion with Stu I, and 1.2 units of T4 DNA ligase were incubated at 24°C for 10 min with the indicated amount of HCC. Samples were made 50 mM EDTA and 0.5% SDS and electrophoresed through a 1.2% agarose gel. The gel was dried and exposed to radiosensitive film (XAR-5,Kodak).

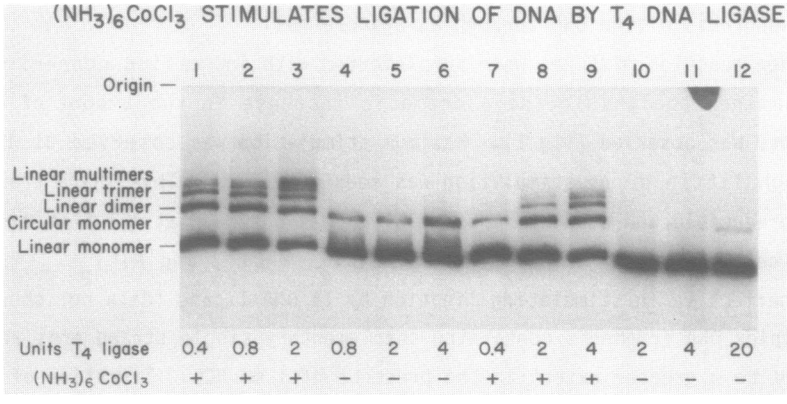

Fig 2. HCC increases the extent of cohesive and blunt end ligation
Reaction mixtures were the same as in fig 1 except lanes 1-6 contained 2.0 nM of Ava II linears of ϕX174 DNA (cohesive ends) and lanes 7-12 contained 1.8 nM Stu I linears (blunt ends). HCC was at 1 mM were indicated. Incubation, gel electrophoresis, and autoradiography were as in fig 1.

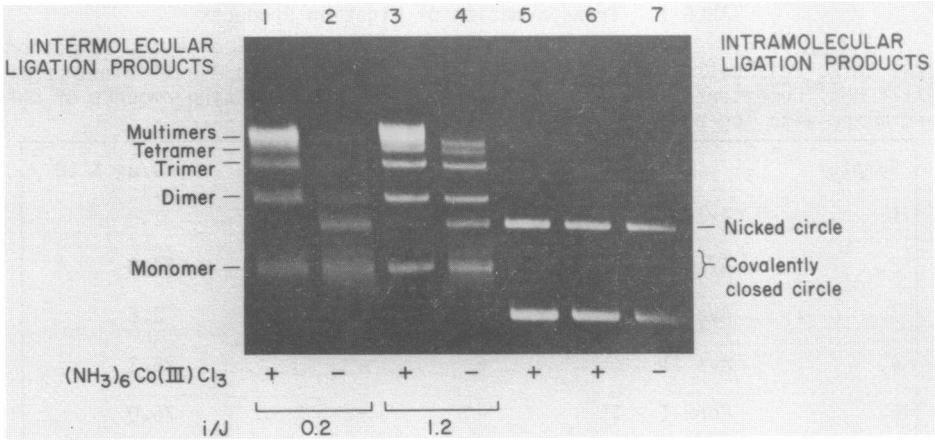

Fig 3. HCC promotes exclusively intermolecular ligation
Standard reaction mixtures were incubated at 24°C for 10 min with 20 units of T4 ligase. An aliquot was electrophoresed through a 0.75% agarose gel and the DNA visualized after soaking the gel in 1 μg/ml ethidium bromide. Lanes 1-4: Ava II linears of ϕX174 DNA(i/J= 0.2 is 4.2 nM); lanes 5-7: a mixture of form I and II DNA. Lane 5 contained no ligase.

stimulated ligation about 50 fold. Previous studies have shown that the extent of ligation is highest at low temperature (21) and this remained unchanged by the presence of HCC (data not shown).

Hexamine Cobalt Chloride Promotes Intermolecular Ligation Events

We next examined the ligation products formed in the presence of HCC. Ligations were done at low DNA concentration to promote intramolecular ligation or high DNA concentration to obtain more intermolecular ligation events. The reaction products were electrophoresed through a 0.75% agarose gel to separate circular monomer (intramolecular) from linear dimer (intermolecular) products (fig 3). When the linear ϕX174 DNA concentration was 4.2 nM (i/J=0.23) the major products of ligation were circular molecules, indicative of intramolecular ligation. At 21 nM DNA (i/J=1.15) at least half the ligation products were linear multimers of full length ϕX174 DNA (fig 3,lane 4). However, when HCC was present at 1 mM all of the ligated DNA was linear dimer or higher multimers regardless of the initial DNA concentration (fig 3,lane 1,3). Ligation in the presence of recA protein also produces exclusively linear multimers (9).

To further demonstrate the decrease in circular ligation products in the

TABLE 1. Transformation of Ligation Products

Aliquots of reactions 1-6 of fig 3 were transfected by the Ca method (23) to E. coli cells (strain HF4714). Sample 7 contained Ava II linears of ϕX174 DNA. Competent cells were transfected with subsaturating amounts of DNA and mixed with log phase cells to visualize phage plaques.

Sample	DNA	Ligase	HCC	PFU/μg X 10^{-3}
1.	AvaII	+	+	1.0
2.	Ava II	+	-	54.0
3.	Ava II	+	+	2.6
4.	Ava II	+	-	25.2
5.	Form I + II	-	+	75.0
6.	Form I + II	+	+	73.0
7.	Ava II	-	-	0.9

presence of HCC we transformed cells with the products of ligation. Since circular DNA molecules transform at 50-100 times the efficiency of linear DNA we could quantitate circular product formation by measuring the transformation efficiency of linear DNA after ligation. Aliquots of the reactions described in fig 3 were transfected into competent cells and plated with indicator cells so phage plaques could be quantitated (table 1). Linear ϕX174 DNA molecules transfect cells and produce a plaque with an efficiency of 1 X 10^5 PFU/μg of DNA. Preincubation with T4 DNA ligase increased the number of plaques over 50 fold but this increase was not observed in the presence of HCC. This shows that circular products of ligation rarely occur (<4%) in the presence of HCC.

Monovalent Ions Alter the Effect of HCC on Ligation

Standard ligation mixtures containing linear ϕX174 DNA with or without HCC were supplemented with various amounts of KCl just prior to the addition of ligase (fig 4). At 20 mM KCl and 1 mM HCC there was a change from exclusively linear multimers to circular ligation products. HCC continued to increase the extent of ligation even in the presence of 100 mM KCl (lane 2 vs 7). When the KCl was added 15 sec after ligase (fig 4, lane 8) and the reaction allowed to proceed 10 min we observed DNA that had electrophoresed to the position expected for circular dimer and trimer ligation products. Further analysis of these DNA ligation products has not been performed. Other

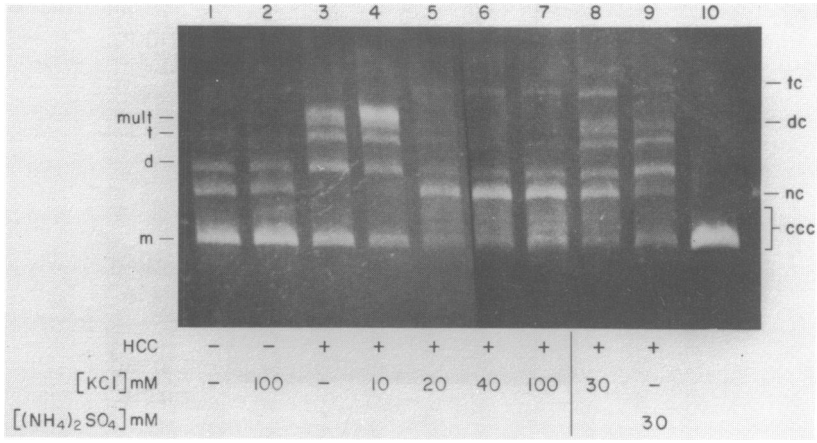

Fig 4. Effect of monovalent ions on T4 ligase action in the presence of HCC
Standard reaction mixtures were supplemented with varying amounts of KCl. DNA was Ava II linears of ϕX174 DNA at 10.6 nM. Lanes 1-9 contained 2 units T4 ligase. In lanes 8 and 9 the indicated salt was added 15 sec after T4 ligase. After 10 min the samples were electrophoresed and the DNA visualized by ethidium staining. Linear ligation products are identified on the left: d,dimer; t,trimer; mult,linear multimers and circular products on the right: ccc,covalently closed circles; nc,nicked circles; dc,dimer circles; tc,trimer circles.

monovalent ions (NaCl and $(NH_4)_2SO_4$) also change the effect of HCC in a similar manner.

Analysis of Ligation Products

We examined the linear multimers formed by T4 ligase in the presence of HCC. Blunt end ϕX174 DNA linearized by digestion with Stu I was ligated by T4 ligase in the presence of HCC and redigested with Stu I. The DNA ligated to linear multimers in the presence of HCC (fig 5, lane 3) was redigested with Stu I and produced exclusively monomer length linears (lane 4).

Because of the sequence assymetry in the 3 base single strand termini of Ava II fragments (5'-G'GACC), T4 ligase does not join these fragments "head to head" under standard reaction conditions. We incubated T4 ligase with Ava II linears and HCC and the resulting linear multimer DNA was purified (see Experimental Procedures). Fig 6 shows the results of redigestion of the linear multimers with Ava II (lane 2). Most of the DNA was cleaved to monomer length linear fragments while partial digestion produced linear dimers and trimers. Complete digestion of the gel purified multimers was obtained with

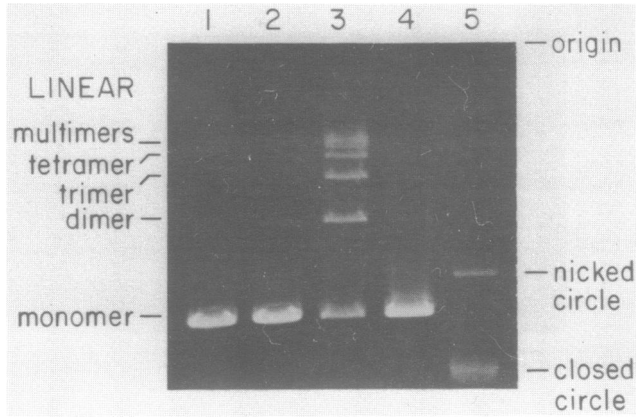

Fig 5. Ligation of blunt end DNA reforms the original restriction site
Blunt end linears (15.9 nM) of ϕX174 DNA produced by digestion with Stu I were used in standard ligase reactions. Tube 3 and 4 contained 1 mM HCC. T4 ligase was added (5 units) to samples 2,3,4 and the mixtures incubated 10 min at 24°. Tube 4 was then brought to 100 mM NaCl and 5 units of Stu I were added. After 1 hr at 37° samples were electrophoresed through an 0.8% agarose gel and the DNA visualized by ethidium bromide staining.

5-fold more Ava II suggesting that complete integrity of the DNA ends was maintained (data not shown).

Multimers were also cut with Xho I which cuts duplex ϕX174 DNA once at a site about 500 basepairs from the Ava II site. Fig 6, lane 3 shows the fragments expected from both internal (linear monomer) and terminal (4800 bp + 500 bp) regions of the linear multimer. Since the linear multimers were at least 4 times monomer length, the 500 bp terminal fragment represents a fraction of the radioactivity (<2%) that is too faint for photographic reproduction. However, the DNA fragment indicative of "head to head" linkage (1000 bp), which could be 10% of the DNA, was not observed. Digestion of multimers with Hae III (lane 4) shows regeneration of Hae III fragment 3 which spans the site originally cut by Ava II and subsequently ligated.

These experiments demonstrate two points: i) the original restriction site is regenerated by T4 ligase catalyzed ligation in 1 mM HCC and ii) the presence of HCC does not alter the T4 ligase requirement that cohesive termini need be complimentary. Other mismatches in terminal sequences were examined and no change in T4 ligase nucleotide specificity was observed by addition of HCC (data not shown).

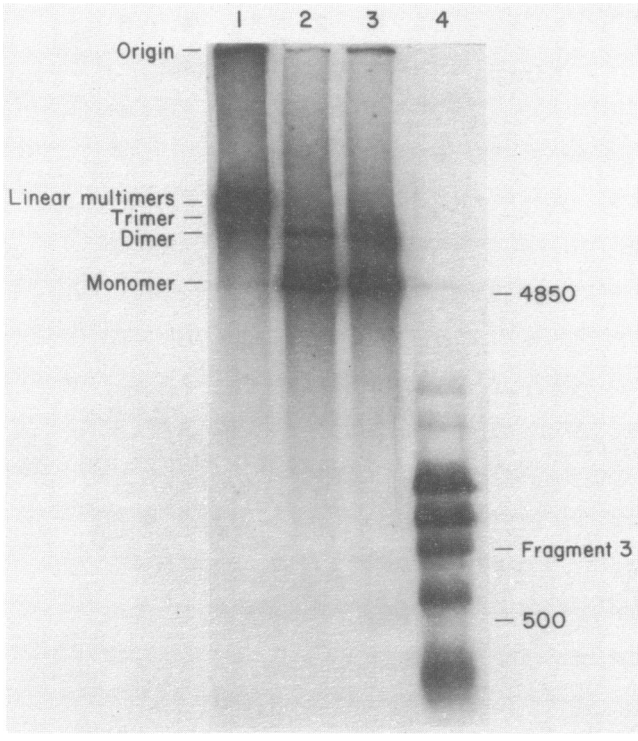

Fig 6. Analysis of linear multimers formed in the presence of HCC
Linear multimers of ϕX174 [^{32}P]DNA formed by ligation in the presence of HCC were purified as described in Experimental Procedures. Lane 1, linear multimers; 2, multimers cut with limiting amount of Ava II; 3, multimers cut with limiting amount of Xho I; 4, multimers cut with Hae III.

Ligation Reactions Involving Synthetic Linkers

To examine the ligation of linker fragments (12 bp) to each other and to large DNA fragments we incubated 5'-end labeled linker DNA, blunt end ϕX174 linear DNA and ligase with or without HCC. Samples were electrophoresed through an 8% polyacrylamide gel and the DNA visualized by autoradiography (fig 7). The ladder of bands are the result of linker-linker ligation events and the increase in band intensity at the origin (lane 4 vs 5) indicates ϕX174-linker DNA ligation events. Other artifacts of linker ligation reactions could also be at the origin of these gels. We observed a slight stimulation of both linker-linker and linker-fragment ligation by 1mM HCC (compare lanes 1 and 3). Other conditions of ligation (eg. pH, ionic strength) were not examined.

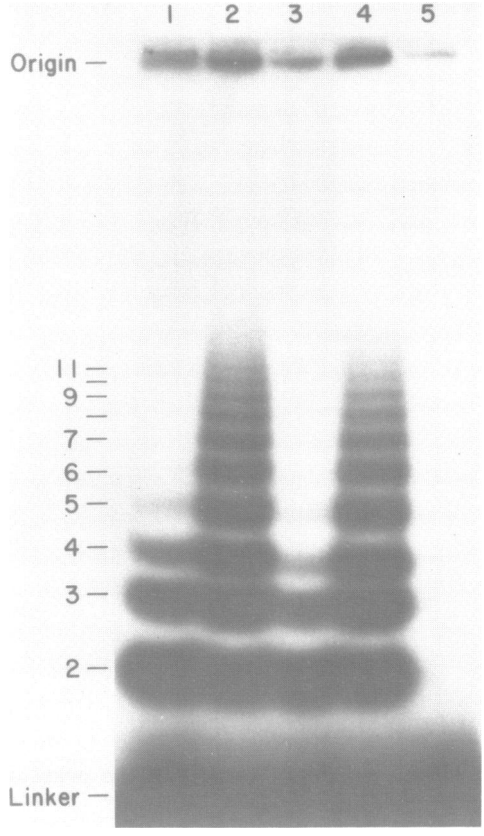

Fig 7. Effect of HCC on Ligation of synthetic linker DNA
Standard ligase reaction mixtures contained 1.8 nM blunt end ϕX174 [^{32}P]DNA and 410 nM of 5'-[^{32}P] linkers (see Experimental Procedures),. HCC was added to sample 1 and 2. Lanes 1,3 contained 20 units of T4 ligase and lanes 2,4 had 80 units of ligase. Ligase was not added to sample in lane 5. Aliquots were electrophoresed through an 8% polyacrylamide gel and the gel was exposed to film.

DISCUSSION

We have presented data showing that hexamine cobalt chloride increases the extent of DNA ligation by T4 ligase. Although no stimulation was observed at 0.5 mM HCC, maximal increase in ligation was seen at 1 mM HCC. Widom and Baldwin (13) observed a similarly sharp transition when measuring light scattering by DNA solutions titrated with HCC. These authors and others (14,22) have ascribed this property to trivalent cation induced condensation

to form side by side aggregates of DNA. It is understandable that a condensation of DNA would stimulate DNA joining by ligase.

Stimulation of ligation was estimated by examining the extent of ligation with varying amounts of enzyme. Cohesive end joining appeared to be stimulated about 5 fold; blunt end joining increased about 50 fold. This increase in ligation allows reactions to be performed at low enzyme and DNA concentrations.

The most striking effect of HCC was to alter the distribution of ligation products. At 1 mM HCC the ligation products were exclusively intermolecular joining events. Circular products of ligation were not observed by electrophoresis or transformation. Even at low DNA concentrations that favor circularization ($i/J<0.1$) all the DNA products were linear multimers. Aggregation to promote exclusively intermolecular ligation also occurs with a protein from Xenopus (8) and with recA protein (9).

HCC induced intermolecular DNA condensation can be reversed with addition of low levels of monovalent ions (13). We found that low levels of NaCl or KCl changed the effect of HCC on DNA ligation. While stimulation of ligation was still observed, circular molecules became a significant portion of the ligation products. When KCl was added 15 sec after T4 ligase and the reaction continued, we observed dimer and trimer length circles. Therefore reaction conditions can be adjusted to obtain first intermolecular then intramolecular ligation without changing the amount of DNA and the ligation reaction is complete in 10 min.

Ligation of linker DNA either to itself or to large DNA fragments was only slightly stimulated by HCC. This may reflect a size limit for trivalent metal induced bridging of polynucleotides to form aggregates. A ligation buffer containing HCC and spermidine was previously recommended to increase efficiency of linker ligation reactions (10). We have not examined ligation reactions in the presence of spermidine.

The joining of DNA fragments is a basic step common to all cloning strategies. The properties of HCC stimulated ligation are useful in the construction of recombinant DNA molecules: 1) blunt end DNA fragments are ligated rapidly and efficiently; 2) exclusively intermolecular ligation events are possible even at low DNA concentrations; 3) the reaction mixture can be rapidly altered to produce circular ligation products; 4) the products of

ligation can be used directly to transform competent cells. For example, these properties could be exploited to clone DNA available only in small quantities.

Acknowledgements

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