present. It is obviously impossible to obtain a unique conformation (which in any case does not exist) from one such measurement. One can attempt to find *possible* conformations which will serve as guides for the types of species present in solution. These conformations can be used to help understand the experimental data and their dependence on base sequence, temperature, solvent, etc. We have found on the basis of semiempirical energy calculations that two base–base-stacked conformations ($I \simeq g^-g^-$ and II) and one base–sugar-stacked conformation (III) should be added to the previously used conformations of g^-g^- and g^+g^+ (slightly base–base stacked) and g^+t and tg^+ (open).

Acknowledgments

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Cloning of Synthetic Deoxyribonucleic Acid That Codes for Embryonic Cardiac Myosin Light-Chain Polypeptide†

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ABSTRACT: Double-stranded complementary deoxyribonucleic acid (cDNA) transcribed in vitro from a partially pure myosin light-chain messenger ribonucleic acid (mRNA) of the chick embryonic heart was cloned in *Escherichia coli* strain χ1776 by using the *Hin*dIII cleavage site in the plasmid pBR322. The insertion of essentially full length DNA was achieved by repeated selection of large-size cDNA transcripts. Of the 12 transformants that contained large-size DNA inserts, the clone pML10 insert was 950 base pairs in length, almost the same size as myosin light-chain mRNA (980 nucleosides). The clone pML10 was identified by hybridization with a highly pure cDNA probe and by hybrid-arrested translation assay. pML10 was further characterized by partial restriction enzyme mapping. The availability of a cloned DNA probe for myosin light-chain facilitates the analysis of the mechanism underlying the induction of cardiac muscle specific gene transcription in presumptive heart-forming cells of the chick blastoderm.

Heart muscle formation, which is an early event during chick embryonic development (Romanoff, 1960), is an attractive model for studying the molecular basis of induction of early embryonic gene functions. The apparently homogeneous population of cells, located on the lateral sides adjacent to Hensen's node in the postgastrulation stage [stage 5, Hamburger & Hamilton (1951)] of the chick embryo, is already programmed to develop into cardiac myoblasts which differentiate eventually into cardiac muscle tissue (DeHaan et al., 1970; Rosenquist, 1970). The availability of these primitive heart-forming cells offers a unique opportunity to probe into the mechanism of gene expression in the early embryo with the aid of cloned DNAs.

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Myogenesis is characterized by the production of large quantities of myosin heavy-chain (MHC) and myosin light-chain polypeptides (MLC_1 and MLC_2) among other proteins (Coleman & Coleman, 1968). Specific differences in both composition and content of MLC subunits occur in a variety of muscle systems (Lowey & Risby, 1971; Sarkar et al., 1971; Yaffe & Dym, 1972; Low et al., 1971; Sreter et al., 1975), and therefore this protein can be used as a marker for monitoring muscle development. In a recent communication (Arnold & Siddiqui, 1979), we have described the isolation, purification, and characterization of MLC mRNAs. In parallel, we have purified MHC mRNA and the cardiac inducer RNA, 7S CEH-RNA, from the chick embryo (Deshpande et al., 1977, and unpublished results). The latter appears to play a crucial role in control of embryonic heart muscle development (Deshpande et al., 1977; Deshpande & Siddiqui, 1977, 1978; Arnold et al., 1978). The respective cDNAs for these RNAs have also been prepared and characterized [Arnold & Siddiqui (1979), and unpublished experiments]. We undertook the amplification of cDNA probes by the molecular cloning technique. It would thus be feasible to monitor and quantitate the synthesis of specific RNAs throughout the period of embryonic development, ranging from the early blastodermal stages to the stage where highly differentiated heart myofibrillar structures are formed. The cloned cDNAs would also allow the study of structure, organization, and expression of cardiac muscle specific genes in the chick embryo.

In the following, we describe the cloning of MLC_2-specific DNA sequences synthesized by using an RNA fraction enriched in MLC_2 mRNA. The insertion of an almost complete DNA segment complementary to MLC_2 mRNA was achieved by repeated selection of large-size cDNA transcripts of MLC_2 mRNA. The clones carrying the MLC_2 gene were identified by hybridization with a highly pure cDNA probe for MLC_2 mRNA and by hybrid-arrested translation assay.

A preliminary account of this work has appeared (Jakowlew et al., 1979).

Materials and Methods

Isolation of mRNA. Hearts were excised from the 16-day-old chick embryos, washed, and stored at −70 °C as described earlier (Arnold & Siddiqui, 1979). RNA was extracted by successive phenol–chloroform extractions of the Pronase-treated homogenate (Monson & Goodman, 1978) of the heart tissue. The poly(A)-containing RNA was obtained from the total RNA by two to three passages through an oligo(dT)–cellulose column (T3) as described earlier (Arnold & Siddiqui, 1979). The RNA was then fractionated by centrifugation on a 5–22% linear sucrose gradient in 70% formamide at 20 °C. The RNA fractions sedimenting between tne marker 18S rRNA and the top of the gradient, which contained MLC mRNA activity upon translation in rabbit reticulocyte lysate (see below), were recovered by ethanol precipitation. The RNA was then recentrifuged on a 15–33.9% isokinetic sodium dodecyl sulfate ($NaDodSO_4$)–sucrose gradient in 10 mM Tris-HCl (pH 7.4), 5 mM EDTA, and 0.5% $NaDodSO_4$. Aliquots from the fractions were again analyzed for translational activity as above.

Cell-Free Translation in Rabbit Reticulocyte Lysate. RNA was translated in cell-free rabbit reticulocyte lysate after treatment with micrococcal nuclease according to Pelham & Jackson (1976) and as described previously (Arnold & Siddiqui, 1979). Five-microliter aliquots from the translation mixture were analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis (Laemmli, 1970), and the gel was examined by fluorography (Boner & Laskey, 1974). Purified myosin prepared from homologous tissue (Wikman-Coffelt et al., 1973) and other marker proteins were run on the same gel as a reference. For quantitation, the preflashed film was underexposed and scanned with an Ortec 4310 densitometer. The translation products were also identified by specific immunoprecipitation using purified antibodies (Arnold & Siddiqui, 1979).

Synthesis of Double-Stranded cDNA. Single-stranded cDNA for MLC mRNA was first synthesized as described previously (Arnold & Siddiqui, 1979), using 400 units/mL avian myeloblastosis reverse transcriptase (supplied kindly by J. W. Beard, Life Sciences, Inc., St. Petersburg, FL) in a 50-μL reaction mixture containing 50 mM Tris-HCl (pH 8.3), 10 mM $MgCl_2$, 0.5 mM dithiothreitol, 4 mM sodium pyrophosphate, 1 mM each of dATP, dGTP, and dTTP, 0.2 mM [^{3}H]dCTP (final sp act. 0.2 Ci/mol), and 10 μg/mL oligo-$(dT)_{12-18}$. The reaction, after incubation for 60 min at 46 °C, was terminated by the addition of 20 mM EDTA and 0.2% $NaDodSO_4$. The mixture was then deproteinized by phenol–chloroform extraction, and the unincorporated nucleotides were removed by gel filtration on a Sephadex G-50 column in 10 mM Tris-HCl (pH 7.5) and 1 mM EDTA. The excluded material was digested overnight with 0.3 N NaOH at 37 °C to remove the RNA template, and the cDNA was recovered by ethanol precipitation in the presence of carrier RNA.

The second strand was synthesized with AMV reverse transcriptase by utilizing the self-priming activity of the single-stranded cDNA according to Monahan et al. (1976). One hundred microcuries of [^{3}H]dCTP (5.5 Ci/mmol) dried by lyophilization was resuspended in 100 μL of buffer containing 50 mM Tris-HCl (pH 8.3), 8 mM $MgCl_2$, 400 μM each of dATP, dGTP, and dTTP, 100 μM [^{3}H]dCTP, 20 mM β-mercaptoethanol, and 20 μg/mL cDNA. The mixture was incubated for 4 h at 46 °C in the presence of 200 units/mL reverse transcriptase. The reaction was stopped and the ds-cDNA was recovered as above.

S_1 Nuclease Digestion of ds-cDNA. S_1 nuclease was isolated from amylase (Vogt, 1973), and the conditions for digestion of single-stranded DNA were optimized. Routinely, the digestion was done for 30 min at 37 °C in 500 μL of buffer containing 30 mM sodium acetate (pH 4.5), 300 mM NaCl, and 4.5 mM $ZnCl_2$. The DNA was recovered by three phenol–chloroform extractions followed by ethanol precipitation and was examined on an alkaline isokinetic 15–30.9% sucrose gradient.

Construction of Hybrid DNA Molecules. Poly(dA) was added to the 3′ termini of ds-cDNA by using calf thymus terminal transferase (Roychoudhury et al., 1976). cDNA (2×10^{-8} termini) was incubated with the enzyme (80 units/mL) in a 50-μL reaction mixture containing 100 mM potassium cacodylate (pH 7.0), 1 mM $CoCl_2$, 0.1 mM [^{3}H]ATP (sp act. 10 800 cpm/pmol), and 0.1 mM DTT for 10 min at room temperature. Under these conditions, 80 dAdo residues were added per DNA chain (average molecular weight for DNA 500 000). The tailed DNA was then fractionated on an isokinetic sucrose gradient under nondenaturing conditions, and fractions representing 800–1000 base pairs in length were pooled and recovered as above. The plasmid pBR322 was linearized by digestion with the restriction endonuclease *Hin*dIII (Boliver et al., 1977), and tailing of linearized plasmid was done with [^{3}H]dTTP as above. The tailed plasmid DNA (200 ng) and the tailed ds-cDNA (50 ng) were heated at 55 °C for 10 min in a 100-μL reaction mixture containing 10 mM

Tris-HCl (pH 8.0), 0.1 M NaCl, and 1 mM EDTA. The DNA was annealed by incubation for 1 h at 46 °C. The water bath was then shut off and the sample allowed to cool slowly at room temperature.

Transformation and Identification of Clones. The hybrid plasmid DNA molecules were then used to transform *Escherichia coli* strain χ1776 (kindly supplied by Dr. R. Curtiss) (Norgard et al., 1978). The tetracycline-resistant clones were grown on sterile nitrocellulose filters placed on top of LB agar plates containing 26 μg/mL ampicillin. After incubation for 24 h at 37 °C, colonies were examined for the inserted DNA sequences according to Grunstein & Hogness (1975) by hybridization with 100 000 cpm [^{32}P]cDNA for MLC_2 mRNA after presoaking the filters in buffer containing 20 μg/mL poly(U) and carrier tRNA.

Sizing and Restriction Analysis of Cloned Plasmid DNA. *E. coli* χ1776 cells were grown to 0.5 A_{550} in L-Broth (Lennox, 1955). Chloramphenicol was added to a final concentration of 12.5 μg/mL. Cells were harvested and lysed (Katz et al., 1973) following chloramphenicol treatment (12.5 μg/mL), and DNA was isolated and purified by cesium chloride–ethidium bromide equilibrium centrifugation (Bazaral & Helinski, 1968). The plasmid DNA was analyzed by electrophoresis on 1 or 2.5% agarose horizontal slab gels prepared in 40 mM Tris–acetate (pH 8.0), 5 mM sodium acetate, and 1 mM EDTA (McDonell et al., 1977). Electrophoresis was performed for 5 to 6 h at 80–100 V at room temperature. Plasmid DNA bands were located under UV light after staining with ethidium bromide. DNA was also analyzed after digestion with restriction endonucleases, and fragments containing the sequences specific for MLC_2 mRNA were located by hybridization with [^{32}P]cDNA as above after the plasmid DNA was transferred to nitrocellulose filters according to Southern (1975).

Cell-Free Translation of Hybrid-Arrested mRNA. One microgram of plasmid DNA (clone pML10) was digested with *Eco*RI and hybridized with 1 μg of total poly(A) RNA from chick embryonic heart tissue (Hastie & Held, 1978). The hybridized samples after ethanol precipitation were translated in rabbit reticulocyte lysate as above.

The cloning work was carried out in the P-3 containment facility according to National Institutes of Health guidelines.

Results

We recently described the isolation of pure mRNAs for cardiac myosin light-chain subunits, MLC_1 and MLC_2, from the chick embryo by immunoadsorption of total polysomes to MLC-specific antibodies (Arnold & Siddiqui, 1979). Under the conditions used the mRNAs were copurified and their purity was assessed by electrophoretic separation, translation, and hybridization assays. Although the MLC mRNAs thus obtained were highly pure, the yield was less than that obtained routinely by the procedure employed here which appears to effectively eliminate nuclease activity. Poly(A)-containing RNA, obtained by two or three cycles of oligo(dT)–cellulose chromatography of total RNA, was fractionated by two successive centrifugations, first on a linear formamide-containing sucrose gradient followed by a second centrifugation on an isokinetic sucrose gradient in $NaDodSO_4$. This yielded fractions which were remarkably pure with respect to MLC mRNA activity tested in the nuclease-treated rabbit reticulocyte lysate. As shown in Figure 1, fraction 12 contained predominantly the mRNA for the smaller of the two myosin light chains, MLC_2, and fraction 11 had the activity for both MLC_1 and MLC_2 polypeptides, although it was relatively free of other contaminating mRNA activity. For the purpose of reverse transcribing and cloning, we used the mRNA in fraction 12.

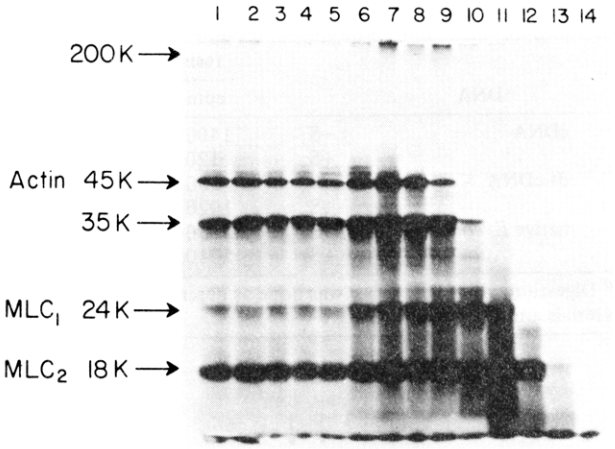

FIGURE 1: $NaDodSO_4$–polyacrylamide gel electrophoresis of translation products of poly(A) RNA fractions. Twenty A_{260} units of poly(A) RNA recovered from the sucrose gradient fractions sedimenting between the 16S rRNA marker and the top of gradient was run on an isokinetic $NaDodSO_4$–sucrose gradient as described under Materials and Methods. RNA in each fraction was ethanol precipitated and redissolved in 100 μL of H_2O. A 1-μL aliquot from each fraction was used for translation in the rabbit reticulocyte lysate system, and a 5-μL aliquot of the mixture was examined on 10% polyacrylamide gel containing $NaDodSO_4$ along with marker proteins as before (Arnold & Siddiqui, 1979). The gel was developed for fluorography as described under Materials and Methods.

Table I: ds-cDNA Synthesis[a]

reaction	av chain length (nucleotides)	yield (μg)	efficiency (%)
cDNA (prepared from 2 μg of mRNA)	950 ± 50	0.8	40
ds-cDNA synthesis[b]	900 ± 50[d]	0.48	60
ds-cDNA (after tailing reaction[c])	800 ± 50[d]	0.28	30

[a] The conditions for cDNA, ds-cDNA synthesis, and the tailing reaction are described under Materials and Methods. [b] Calculated from the extent of incorporation of [^{3}H]dCTP of specific activity 25-fold higher than that used for the first strand cDNA synthesis (see Materials and Methods). [c] Calculation for the efficiency of the tailing reaction was based on the fraction recovered after sucrose gradient centrifugation of the reaction mixture. Only the fractions migrating close to the original cDNA were used; the remaining one-third of the material sedimenting slower than cDNA was discarded. [d] Base pairs.

The efficiency of cDNA synthesis for MLC_2 mRNA was good (40%; see Table I), and the average size of the product was ~950 nucleosides when examined on an alkaline sucrose gradient. The molecular sizes of MLC_1 and MLC_2 mRNAs were previously estimated to be 1090 and 980 nucleosides, respectively (Arnold & Siddiqui, 1979). It appears, therefore, that almost complete chains of cDNA for MLC_2 mRNA were the predominant product of reverse transcription. The cDNA was further enriched in large-size transcripts by selecting a fraction of the gradient which eliminated slower sedimenting cDNA fragments. The cDNA was then converted to a double-stranded cDNA by a second incubation with AMV reverse transcriptase. About 60% of the self-priming cDNA was converted to double-stranded molecules. The unused single-stranded cDNA and the "hairpin" loops were digested by incubation with S_1 nuclease under conditions optimized for single-stranded DNA digestion. As indicated in Table II, virtually all of he radioactivity of ds-cDNA remained acid insoluble, whereas almost 92% of the label in single-stranded cDNA became acid soluble. When examined on an alkaline

Table II: S_1 Nuclease Resistance of ds-cDNA[a]

DNA		resistance to S_1 cpm	%
cDNA	$-S_1$	1400	
	$+S_1$	120	8.5
ds-cDNA	$-S_1$	950	
	$+S_1$	1020	107
native *E. coli* DNA	$-S_1$	5200	
	$+S_1$	5040	97

[a] Digestion with S_1 nuclease was done as described under Materials and Methods.

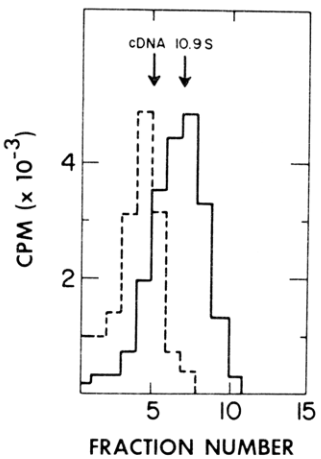

FIGURE 2: Sizing of double-stranded [^{3}H]cDNA on an alkaline sucrose gradient. [^{3}H]dCTP-labeled ds-cDNA before and after digestion with S_1 nuclease was centrifuged on an alkaline 10–30.9% sucrose gradient in 0.1 M NaOH, 5 mM EDTA, and 0.9 M NaCl. Radioactivity in each fraction, after neutralization with acetic acid, was counted in an Aquasol scintillation mixture. Single-stranded cDNA synthesized from the same mRNA and $\theta\chi$174 DNA (Miles) were used as markers.

sucrose gradient (Figure 2), the ds-cDNA after digestion with S_1 nuclease sedimented almost identical with the undigested single-stranded cDNA, suggesting that the hairpin loops of ds-cDNA were effectively removed by S_1 nuclease and no overdigestion of the ds-cDNA occurred.

Approximately 80 dAdo residues were then added to the 3′ termini of ds-cDNA by using terminal deoxynucleotidyl transferase. The average length of the tailed ds-cDNA was ~800 ± 50 base pairs. For elimination of short ds-cDNA molecules which resulted due to possible nuclease contamination of the enzyme, the portion of tailed cDNA sedimenting close to the original untailed ds-cDNA was pooled and used for cloning. The 30% efficiency for the tailing reaction (Table I) was determined based on the recovered two-thirds material of tailed ds-cDNA used for the reaction; the remaining one-third portion which sedimented slower than the original preparation was discarded. The bacterial plasmid pBR322 was cleaved with *Hin*dIII at the single tetracycline resistance site (Bolivar et al., 1977). The linearized plasmid was then tailed with ~80 dT residues as above. Equimolar amounts of ds-cDNA and the plasmid DNA were annealed, and the resulting chimeric plasmids were used to transform *E. coli* χ1776. Thirteen clones that were tetracycline sensitive were obtained from annealing 50 ng of ds-cDNA and 200 ng of the plasmid DNA.

For identification of clones carrying MLC_2 gene specific sequences, the tetracycline-sensitive clones growing on nitrocellulose filters were screened by means of hybridization with [^{32}P]cDNA prepared by using a pure MLC_2 mRNA. The MLC_2 mRNA was prepared by successive sucrose gradient centrifugations, as above, of poly(A) RNA obtained by immunoadsorption of total polysomes to MLC-specific antibodies (Arnold & Siddiqui, 1979). The purity of MLC_2 mRNA was assessed by criteria described earlier (Arnold & Siddiqui, 1979). Figure 3 shows the autoradiogram of colonies after hybridization with the cDNA to MLC_2 mRNA. Of the total clones examined, 12 exhibited strong hybridization response; clone pML3 was a recombinant background clone which was tetracycline resistant. The hybridization was specific since colonies pML13, pML14, and pML15 carrying control plasmid pBR322 DNA alone did not hybridize under the same conditions. Digestion of clones pML7, pML8, pML9, and pML10 plasmid DNA with *Hin*cII endonuclease generated two fragments which were readily separated upon electrophoresis on an agarose gel (Figure 4). The smaller fragments in each case contained the MLC_2 sequence as shown by their ability to hybridize with [^{32}P]cDNA for MLC_2 mRNA. The positive response in the slower migrating band for clone

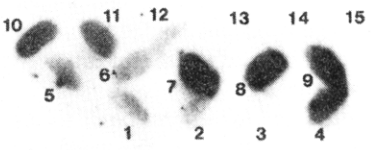

FIGURE 3: Hybridization of MLC_2 [^{32}P]cDNA to colonies of transformed *E. coli* strain χ1776. Colonies were obtained by streaking out plasmid-containing clones on a nitrocellulose filter and incubating them on top of an LB agar plate for 24 h at 37 °C. Denaturation of the colonies and hybridization with the probe [^{32}P]cDNA for pure MLC_2 mRNA were done according to Grunstein & Hogness (1975). Clones pML13, pML14, and pML15 were plasmid pBR322 containing *E. coli* χ1776 used as a control.

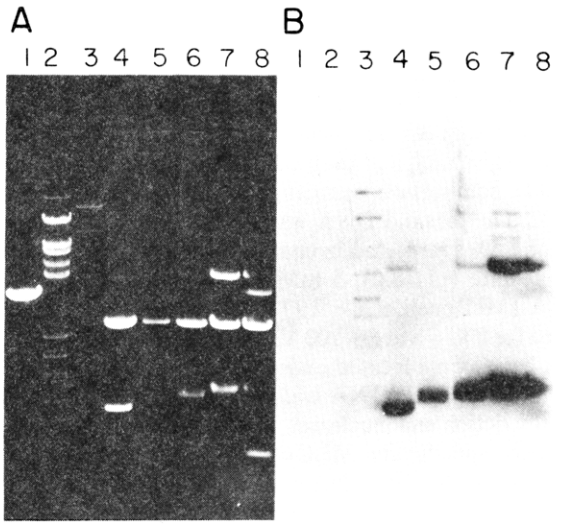

FIGURE 4: Sizing of cloned MLC_2 DNA inserts. Plasmid DNA (1 μg each) from clones pML7, pML8, pML9, and pML10 was digested with 10 units of restriction endonuclease *Hin*cII (Biolabs) for 12 h at 37 °C. The products were separated by electrophoresis in 1.5% agarose gel as described under Materials and Methods. The gel was stained with ethidium bromide and examined under UV light (panel A). Panel B shows the autoradiograph of the DNA digests after hybridization with ^{32}P-labeled cDNA for pure MLC_2 mRNA. The slow-moving bands in lanes 6 and 7 are the undigested plasmid DNA. The partial bands in lane 8, which contains the control plasmid pBR322, are due to spillover material from lane 7. The faint bands at the top are attributable to uncut supercoiled or nicked molecules of monomeric or dimeric plasmids. Lane 1, *Eco*RI digest of pBR322 DNA; lane 2, *Eco*RI and *Sma*I digests of λrif^{d}18 DNA (Murray & Murray, 1975); lane 3, ^{3}H-labeled λDNA digested with *Hin*dIII (Lindahl et al., 1977); lanes 4–7, DNA from clones pML7, pML8, pML9, and pML10 digested with *Hin*cII; lane 8, *Hin*cII digest of pBR322.

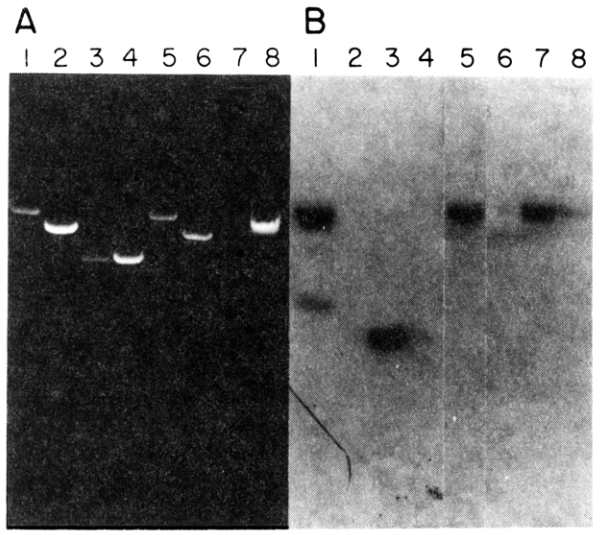

FIGURE 5: Restriction digestion of cloned plasmid pML10 DNA and hybridization with [^{32}P]cDNA. Clone pML10 DNA was digested with restriction endonucleases (see below), and the products were examined by 1% agarose gel electrophoresis after staining with ethidium (panel A) and by autoradiography (panel B) after hybridization with the probe ^{32}P-labeled cDNA for pure MLC_2 mRNA. Lanes 1 and 2, *Eco*RI digests of pBR322 DNA and clone pML10 DNA, respectively; lanes 3 and 4, *Hin*cII digests of pBR322 and clone pML10 DNA, respectively; lanes 5 and 6, *Sal*I digests of pBR322 and clone pML10 DNA, respectively; lanes 7 and 8, *Pst*I digests of cloned and pBR322 DNA, respectively.

pML10 (lane 7, panel B) represents the undigested plasmid DNA. By comparing the migration rates of smaller fragments generated by digestion of the control plasmid pBR322 and those of marker DNAs of known molecular weights [lanes 1 and 2 of panel A and lane 3 of panel B; see Lindahl et al. (1977) and Murray & Murray (1975)], we estimated that the length of the MLC_2 gene sequence in clone pML10 is 950 ± 50 nucleotides, including the poly(dA·dT) tails. Our estimate of the length of dA and dT residues of 80 bases each calculated from the specific activities would suggest that the net size of the inserted DNA is ~790 ± 50 base pairs, roughly the same size as the ds-cDNA used here. Preliminary restriction analysis utilizing several other endonucleases also confirmed the size of the insert MLC_2 DNA in clone pML10. To exclude the possibility of artifacts in colony hybridization and to ascertain that the clone does indeed carry the MLC_2 gene sequence, we digested the clone pML10 DNA along with the plasmid pBR322 DNA with endonucleases *Eco*RI, *Hin*cII, *Sal*I, and *Pst*I and hybridized them with ^{32}P-labeled cDNA for MLC_2 after a Southern transfer on nitrocellulose paper following agarose gel electrophoresis as shown in Figure 5. Clearly, the probe [^{32}P]cDNA hybridized to the clone pML10 DNA in each case and not to the control plasmid DNA, as expected. Furthermore, none of these enzymes appeared to cleave within the insert DNA sequences. Restriction endonucleases *Hae*III, *Bst*II, and *Alu*I, however, cleaved within the insert DNA at several positions (data not shown).

The fidelity of the MLC_2-specific gene insert in pML10 was further documented by the hybrid-arrested translation assay (Figure 6). This assay is based on observations that when a mRNA is hybridized to its corresponding cDNA the translation of the mRNA is specifically arrested. Clone pML10 plasmid DNA was first digested with *Eco*RI, and the product after heat denaturation was hybridized in formamide with total poly(A) RNA from the chick embryonic heart. *Eco*RI-treated control plasmid pBR322 DNA was hybridized with another batch of the same RNA under identical conditions. As seen in Figure 6, the translation activity of clone pML10 DNA containing the mRNA fraction was identical with the mRNA to which control plasmid DNA was added with the exception of one polypeptide that was identical with MLC_2. Mock hybridization of total mRNA in the absence of the added DNA did not affect its translational activity. The synthesis of MLC_2 polypeptide was reduced by more than 75%. The residual counts in the MLC_2 band might be due to incomplete hybridization. The specific neutralization of the synthesis of one polypeptide (MLC_2) nevertheless is a clear demonstration for the qualitative presence of the MLC_2-specific DNA sequence in the clone pML10 plasmid DNA.

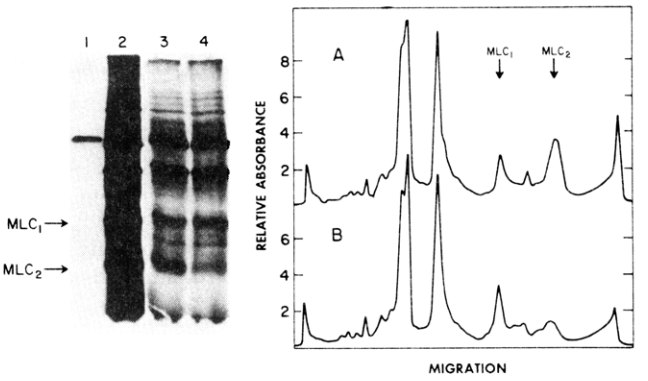

FIGURE 6: Cell-free translation products of control and hybrid-arrested poly(A) RNA. Clone pML10 DNA and plasmid pBR322 DNA after digestion with *Eco*RI were hybridized with total poly(A) RNA of the 16-day-old chick embryonic heart as described under Materials and Methods. The material after recovery by several ethanol precipitations was subjected to the cell-free translation assay in the rabbit reticulocyte lysate as before (Arnold & Siddiqui, 1979). The products were analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis and examined by fluorography. Lane 1, micrococcal nuclease treated lysate; lane 2, translation products of total poly(A) RNA; lane 3, translation products of total poly(A) RNA after incubation with pBR322 DNA; lane 4, translation products of total poly(A) RNA after hybridization with clone pML10 DNA. Panels A and B represent the densitometric tracings of lanes 3 and 4, respectively.

Discussion

The main aim of these experiments was to isolate a hybridization probe that can be used to analyze the early embryonic gene expression specific to cardiac muscle development. We have chosen MLC as a marker protein since specific changes are known to occur in MLC polypeptides during muscle development. Our approach was to synthesize DNA sequences complementary to a rapidly isolatable partially pure mRNA for either of the two MLC polypeptides and amplify the cDNA by cloning using an appropriate host–vector system. The results reported here indicate that amplification of essentially a full-length DNA insert complementary to pure MLC_2 mRNA was achieved with high fidelity. Cloning of RNA sequences for other marker muscle proteins and of 7S CEH-RNA is currently in progress.

The procedure employed in these studies for isolation of MLC_2 mRNA (and for MHC mRNA, which will be reported elsewhere) was rapid and provided functionally intact mRNAs in high yields. Although we have previously obtained pure MLC mRNAs by using immunoadsorption of polysomes, the high level of ribonuclease activity in the heart tissue precluded isolation of intact MLC mRNA on a routine basis. On the basis of the translation assay, the RNA contained in fraction 12 from the second sucrose gradient centrifugation was highly enriched in MLC_2 mRNA activity. No other labeled polypeptides were discernible on 12% polyacrylamide gels. The cDNA synthesized against fraction 12 mRNA had an average

chain length of 950 ± 50 nucleosides, almost the same size as MLC_2 mRNA (980 nucleosides). The cDNA was further enriched for large-size transcripts in order to eliminate the possible contamination of low molecular weight mRNA products. The second cDNA size selection after the tailing reaction was essential since terminal transferase apparently contains endonucleolytic activity (Humphries et al., 1977). The cDNA was readily converted to ds-cDNA by using AMV reverse transcriptase, and digestion of ds-cDNA with S_1 nuclease under chosen conditions rendered intact ds-cDNA without the hairpin loops.

The fidelity of the procedure is documented by hybridization of the cloned DNA fragments with a pure cDNA for MLC_2 mRNA and by hybrid-arrested translation assay. The data indicated that sizable MLC_2-specific gene sequences were present in all clones tested, and the maximum insert was 800 ± 50 base pairs long. On the basis of the size estimate of MLC_2 mRNA (Arnold & Siddiqui, 1979), there are ~340 extra nucleosides in the mRNA than required to code for MLC_2 polypeptide. On the assumption that most of these noncoding nucleosides are located in the 3′ terminus, more than half of the coding region of MLC_2 mRNA is still represented in the clone pML10 plasmid DNA. Hybridization of cloned DNA fragments with total poly(A) RNA and its subsequent translation unambiguously identified the presence of MLC_2-specific DNA sequence in the clone DNA. Higher concentrations of both the clone as well as the control plasmid DNAs (more than 1 $\mu g/\mu g$ of mRNA) produced nonspecific inhibitory effects on translation. cDNA and plasmid DNA are known to cause nonspecific inhibition of translation, possibly by interacting with some component other than mRNA (Hastie & Held, 1978).

In several developmental systems, the increased synthesis of specific proteins, such as hemoglobin, ovalbumin, and fibroin, has been shown to be transcriptionally controlled (Ross et al., 1974; McKnight et al., 1975; Harris et al., 1975; Suzuki & Suzuki, 1974). Myogenesis involves dramatic changes in protein synthesis which are probably due to alteration in specific mRNA population (Paterson & Bishop, 1977). There is some evidence that the mRNA coding for MHC in skeletal muscle cell cultures appears in an inactive form in dividing myoblasts, suggesting that the synthesis of this protein is regulated posttranscriptionally (Buckingham et al., 1976; Morris et al., 1972). Others, however, have shown that high concentrations of several mRNAs accumulate in cultures developing toward myofibril formation (Paterson & Bishop, 1977; Strohman et al., 1977; Devlin & Emerson, 1979), which correlates with their capacity to translate large amounts of myogenic proteins. The change from myoblast to myofibril parallels the appearance of a new class of abundant mRNAs (Paterson & Bishop, 1977). Similar information with respect to cardiac muscle development is not available, but, more importantly, the nature of transcriptional activity in situ in embryonic cells prior to myoblast formation is totally unknown. The availability of cloned DNA probes specific for developmentally regulated marker macromolecules would thus facilitate our analysis of the mechanisms underlying the induction of embryonic cardiac muscle gene transcription.

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Mechanism of the Effect of Organic Solvents and Other Protein Denaturants on Neocarzinostatin Activity†

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ABSTRACT: Several alcohols and other organic solvents enhance the in vitro DNA cutting activity of neocarzinostatin (NCS). Hexamethylphosphoramide is the most potent of the stimulators tested. Kinetic analysis of the DNA scission by NCS in the presence of 2-propanol shows that the solvent effect is on the V_{max} and not on the K_m. All the solvents, above their optimal levels, inhibit NCS activity, and they also increase the rate of its inactivation on preincubation in the absence of deoxyribonucleic acid (DNA). Among the other protein denaturants, urea stimulates the activity of NCS over a wide range of concentrations. In contrast, guanidine hydrochloride at subdenaturing levels strongly inhibits the reaction. One possible explanation for the stimulation of NCS activity by organic solvents and urea is that by breaking down water-cooperative structures they allow increased motility of the antibiotic to a conformation favorable for reduction of its disulfide bonds to generate an active species of the drug for its interaction with DNA. The possible involvement of the nonprotein chromophore of NCS in the activation process is also discussed. In addition, NCS rendered inactive by various treatments has a lower isoelectric point and specifically blocks the activity of native NCS. These results indicate that there are a limited number of DNA binding sites for which the two forms of NCS compete and provide further evidence that direct interaction between drug and DNA is responsible for its biological effects. Since several of the treatments generating the inhibitory species of NCS also cause chromophore release and since auromomycin-induced scission of DNA is blocked by its nonchromophore-containing form (macromomycin), the possibility is raised that loss of chromophore from the protein generates the inhibitory species.

Neocarzinostatin (NCS[1]), an antitumor antibiotic, is a single-chain, acidic protein of molecular weight 10700 with two disulfide bonds (Ishida et al., 1965; Meienhofer et al., 1972a). Recently, we have shown that NCS possesses a nonprotein chromophore that can be removed by methanol extraction and other techniques (Napier et al., 1979). In addition to its ability to inhibit DNA synthesis (Ono et al., 1966; Homma et al., 1970; Sawada et al., 1974; Beerman & Goldberg, 1977), cause DNA strand breakage (Beerman & Goldberg, 1974; Sawada et al., 1974; Tatsumi et al., 1974; Ohtsuki & Ishida, 1975; Beerman & Goldberg, 1977), and induce DNA repair synthesis in whole cells (Tatsumi et al., 1975; Hatayama & Goldberg, 1979) and in isolated nuclei (Kappen & Goldberg, 1978a), NCS introduces single-strand breaks almost exclusively at thymidylate and adenylate residues in DNA (Poon et al., 1977; Hatayama et al., 1978; D'Andrea & Haseltine, 1978) in vitro in a reaction greatly stimulated by a sulfhydryl compound (Beerman & Goldberg, 1974; Poon et al., 1977; Beerman et al., 1977; Kappen & Goldberg, 1977, 1978b). We have further shown that while mercaptans activate the DNA cutting by NCS, they also inhibit it at high concentrations and rapidly inactivate NCS on preincubation in the absence of DNA (Kappen & Goldberg, 1978c). In addition, several DNA-intercalating drugs (Kappen et al., 1979) and α-tocopherol strongly inhibit the in vitro reaction, but alcohols and other organic solvents greatly stimulate the in vitro reaction (Kappen & Goldberg, 1978c). In an attempt to understand the mechanism of alcohol stimulation of NCS activity, we have studied the effects of several alcohols, other organic solvents, and denaturants on the activity of NCS under different conditions by using linear duplex (λ) or superhelical (pMB9) DNA as the substrate. In addition, we find that NCS rendered inactive by various treatments is able to block the DNA scission reaction of native drug.

Materials and Methods

[*methyl*-^{3}H]Thymidine-labeled λDNA was prepared as described (Kappen & Goldberg, 1977). The plasmid DNA (pMB9) was isolated by gentle lysis of the cells (*Escherichia coli* HMS 174pMB9) with lysozyme and detergent, followed by centrifugation to remove chromosomal DNA (Clewell & Helinski, 1969). Further treatment included banding in a CsCl gradient containing ethidium bromide at 200 μg/mL; the ethidium bromide was subsequently removed with Dowex 50

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[1] Abbreviations used: NCS, neocarzinostatin; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetate.

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