The Isolation of RNA Homologous to the Genetic Control Elements of the Lactose Operon*

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A sequential DNA-RNA hybridization procedure is described whereby RNA homologous to a target DNA region 35 to 140 base pairs in length can be purified up to 6700-fold from a complex in vitro transcript to a homogeneity sufficient for sequence analysis. Requirements for the procedure include: (a) uniform transcription over the target DNA region in vitro; (b) specialized transducing phages which carry genetic deletions defining the target region on either side; and (c) specialized transducing phages which carry the target DNA in opposite orientations. These requirements have been met for the genetic control region (promoter, operator) of the lactose operon of Escherichia coli, to which the method was applied. The procedure is independent of the activity of the genetic control signals under study and can therefore be applied without modification to the study of point mutations introduced into the template.

Genetic control of the lactose (lac) operon of Escherichia coli has been shown to occur at the level of transcription and to involve three DNA-protein interactions: (a) RNA polymerase interacts at the promoter to initiate transcription (1-4). (b) This interaction is under the positive control of the catabolite gene activator protein (CAP) which, in the presence of cyclic adenosine 3':5'-monophosphate (cAMP), stimulates the initiation of lac mRNA synthesis by RNA polymerase 50-fold (5-8). (c) Negative control is exerted by the lac repressor (9, 10) which binds to the operator (11, 12) and decreases transcription initiation of the operon by a factor of 2000 (13) in the absence of inducer, allo lactose (14).

In order to gain some insight into the mechanisms of these remarkably specific and complex interactions, we have developed a technique for determining the DNA sequence of the entire lac genetic control region. The DNA is sequenced by analysis of RNA homologous to the target region, in an extension of the indirect RNA sequencing approach to DNA sequences used by others (15-18). RNA is first transcribed in vitro over the target DNA region utilizing “read-through” transcription (transcription initiated by RNA polymerase at the lac promoter) (18). The RNA is then hybridized in two or more successive steps to the DNA of transducing phages which carry deletions defining the target DNA region on either side (19). A great advantage of the technique is that it does not rely on the physiological activity of the signals under study, such as binding of repressor (17) or transcription initiated at the lac promoter (18). Thus the method allows access to sequences in a promoter which are not normally transcribed (18, 20). Furthermore, it allows isolation of RNA carrying any point mutation of interest, merely by introducing the mutation into the template.

MATERIALS AND METHODS

Strains

The bacterial strains used are listed in Table I, and the bacteriophages used are described in Fig. 1. λptrp-lac F36aS80 was constructed

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TABLE I

<table>
<thead>
<tr>
<th>Strain</th>
<th>Pertinent Characteristics</th>
<th>Source or References</th>
</tr>
</thead>
<tbody>
<tr>
<td>007078</td>
<td>F' Δ(lac-proA)-lacI</td>
<td>Barnes et al. (1974)</td>
</tr>
<tr>
<td></td>
<td>Δ(lac)X74, Su III</td>
<td></td>
</tr>
<tr>
<td>F36a</td>
<td>F' Δ(lac-proA,B)-lacI</td>
<td>Mitchell et al. (1974)</td>
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<td>Δ(trp-tob-lac)-36a, Su-</td>
<td></td>
</tr>
<tr>
<td>X830la</td>
<td>F' lac I Δ(α-&lt;s20&gt;^2)</td>
<td>Eron et al. (1970)</td>
</tr>
<tr>
<td></td>
<td>proc-trp R</td>
<td></td>
</tr>
</tbody>
</table>

Media and Buffers

The media and buffers used are described in Reznikoff et al. (33) and Barnes et al. (19) except for the following: Strand buffer (1 mM Na EDTA/0.1 M K$_2$HPO$_4$/HCl to pH 8.4 to 8.6), DNase buffer (0.05 M and 20 x SSC (3 M NaCl/0.15 M sodium citrate, pH 7.0). The 20 x SSC was filtered through a nitrocellulose filter into a sterile bottle, stored at 4°, and diluted with deionized water to make 2 x SSC immediately before use.

Miscellaneous Procedures

All glassware to come in contact with RNA or ribonucleoside triphosphates was acid washed, dried, and autoclaved by rinsing in a 1 to 2% solution of dichlorodimethylsilane (Alrich Chemical Co., Inc.) in CCl$_4$, followed by baking at 110-150° for 3 hours.

Plastic apparatus, which could not be acid-washed, and metal filter holders were treated with a 1% solution of isocyanate or Alconox containing 1 ml/liter of diethylypyrocarbonate for at least 15 min to inactivate any nuclease (35), followed by thorough rinsing and drying.

Dialysis tubing (size 20, Union Carbide) was boiled three times in 15% potassium carbonate and 3 times in 10-2 M EDTA before being autoclaved in deionized water and stored at 4°.

B-6 nitrocellulose filter membrane was obtained from Schleicher and Schuell as sheets (35 x 55 cm), from which 2.5-cm circles were cut. Phenol was prepared by distillation into water. It was stored at -20°.

Growth of Phage

λ super-α and λα-α/lac phage were grown by the large scale plate lysate procedure described in Reznikoff et al. (33), except that the α0 of the bacterial suspension was 0.02 to 0.14 after adsorption of phage and dilution into broth for incubation. λα0 placl and α0 pla65 were grown as described by Reznikoff et al. (33), except that λ pla5 was grown in rich medium (LB5). Purification of phage was described previously (33).

Extraction of DNA

Extraction of DNA for templates was carried out as described previously (33), except that the purified phage was diluted to 1 x 10$^8$ to 10 before extraction.

DNA Separated Strands

The strands of transducing phage DNAs were separated using a modified version of the procedure of Szybalski et al. (36), developed by H. Lozeron. One milliliter of phage at 1 x 10$^{12}$ plaque-forming units were dialyzed against 1 liter of strand buffer for 3 to 5 hours at 4°. The phage suspension was then diluted to 1,800 ml with strand buffer, 0.52 ml of poly(U,G) (Miles, lot 4) at 1 mg/ml, 20 µl of 10% sodium Sarkosyl were added, and the mixture was heated for 3 min at 95° and then chilled in an ice bath. Buffer (0.06 ml of 0.5 M Tris-Cl, pH 7.6) and 14 ml of saturated optical grade CsCl (Code G%, Kerr-McGee Chemical Corporation, Chicago) were then added (final density, 1.725 g/ml). The solution was centrifuged in a 1-inch x 3.5-inch polyallomer centrifuge tube (Beckman, preboiled in 0.1 M EDTA, pH 10) at 32,000 rpm in a Spinco 42.1 or 25,000 rpm in a Spinco SW 27 rotor for 60 to 90 hours at 20°. Fractions of 0.3 ml were collected. The outside halves of each strand peak were pooled and self-anneled at 67° for 1 hour before storing at 4° in the CsCl gradient solution. No effort was made to remove CsCl or poly(U,G).

α-32P-Labeled Ribonucleoside Triphosphates

α-32P-rNTPs at a specific activity of 100 mCi/µ mole were prepared by the procedure of Symons (37) as modified by J. Dahlberg. They were stored in 80% ethanol at -20° as a concentration of 1 mCi/ml and used as soon as possible. When stored in this way they retained the ability to be incorporated at an efficiency of at least 50% for up to 10 days.

Carrier RNA

Yeast tRNA (General Biochemicals, Chagrin Falls, Ohio) was dissolved in 0.2 M sodium acetate, pH 5.4, at about 10 mg/ml and treated with 5 µl/ml of DEPC* at 37° for 10 min to inactivate any nucleases. The solution was then filtered through a nitrocellulose filter. Alternatively, the carrier RNA was prepared by phenol extraction. The RNA was precipitated by adding 3 volumes of 100% ethanol. After at least 4 hours at 20°, the RNA was removed from suspension by centrifugation at 10,000 x g for 10 min. After drying under vacuum, the

3H. Lozeron, personal communication.

J. Dahlberg, manuscript in preparation.

The abbreviations used are: DEPC, diethylpyrocarbonate (trade name, Bayovin, Naftone, Inc., Chicago); LAC, RNA transcripts over the lac operon or its genetic control elements in the same direction as lac mRNA; CAL, RNA transcripts over the operon in the opposite direction.

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pellet was resuspended in H2O at a concentration of 10 mg/ml (A260 = 300) and stored frozen until use.

**RNA Polymerase**

For the preliminary portions of the work, RNA polymerase was isolated from *E. coli* B by the procedure of Chamberlin and Berg. The enzyme was centrifuged through a 15 to 30% glycerol gradient containing 0.05 M KCl in a Spinco SW 27 rotor at 25,000 rpm for 24 hours. Some preparations of RNA polymerase prepared in this fashion were found to be very little of the target genetic control region from *pho85lac*. Typical of such transcripts was an increased transcription of *X* homologous sequences at the expense of CAL sequences. Later, RNA polymerase, which consistently transcribed this region from *pho85lac*, was obtained as a gift from R. Burgess (purified after Burgess and Bautz and Dunn [40]).

**RNA Synthesis Protocol**

For protocol A, the 250-μl reaction volume contained 1.5 μl of an α-32P-ribonucleoside triphosphate at a final concentration of 0.06 mM, a 2-fold excess of the other three ribonucleoside triphosphates (0.12 mM), 0.002 M Na2EDTA, 0.013 M MgCl2, 0.15 M KCl, 0.025 M N2-hydroxylpropylpiperazine-N-2-ethanesulfonic acid-NH4 (pH 7.9), 15 μg of DNA polymerase and 2 μg of RNA polymerase. The reaction was incubated at 37°C for 0.5 to 6.0 hours and assayed every 20 to 30 min. Protocols B and C used at earlier stages of this work employed 0.04 M MgCl2 and 0.06 M KCl. In addition, protocol C used 0.04 M Tris-HCl, pH 7.9, instead of N2-hydroxylpropylpiperazine N2 ethanesulfonic acid. Protocol A was substituted for protocols B and C in an attempt to increase the transcription of the target region from *pho85lac* DNA by RNA polymerase purified by the procedure of Chamberlin and Berg [38]. Lowering the MgCl2 concentration from 0.040 to 0.013 M and raising the KCl concentration from 0.06 to 0.15 M had no appreciable effect on the quality of the RNA transcript, but the new salt conditions were adopted because the rate of ribonucleoside triphosphate incorporation was 60% faster. To assay for RNA synthesis (41), 1 μl of reaction mixture was spotted onto a 1-cm square of Whatman No. 1 filter paper which had been soaked in 0.01 M EDTA and dried. The paper was then rinsed twice in 50 to 100 ml of 3% trichloroacetic acid/1% sodium pyrophosphate for 5 min at room temperature. Due to the high levels of radioactivity (107 dpm/sample), it was necessary to use a hand Geiger counter at a standard distance instead of a scintillation counter to measure the trichloroacetic acid-acprecitable counts. When incorporation stopped (usually within 1 hour, 30 to 90% of the input radioactivity having been incorporated), 1 ml of DNase buffer, 100 μg of carrier tRNA, 0.04 M EDTA, 0.01 M Tris-HCl, pH 7.9, and 50 to 100 μg of DNase (Worthington, code DPPF, stored as a 1 mg/ml of solution in 0.002 N HCl) at 4°C were added. Incubation with DNase was carried out at 25-33°C for 10 min. DNase was inactivated by adding 5 μl of DEPC, mixing on a Vortex mixer, and incubating for 10 min at 37°C. The solution was then filtered through a nitrocellulose filter which had been previously rinsed with H2O. The filter was rinsed with 1 volume (1.25 ml) of 0.5 M sodium acetate, pH 5.4. Three volumes (7.5 ml) of cold 100% ethanol were mixed with 0.15 ml of separated DNA strands (at A260 of 2

inactivated the residual RNA. The yield of hybridized RNA was measured by Cerenkov radiation before each filter was removed from the scintillation vial and washed with 50 ml of 9 x SSC from each direction. In an alternative, equivalent RNAase treatment, 4 ml of 2 x SSC + 100 μg of RNAase were added directly to the hybridization vial before filtering. After 10 to 20 min at 37°C, the mixture was filtered through nitrocellulose as before, followed by washing with 50 ml of 2 x SSC and iodateacetate treatment as before.

**DNA Elution—** Each filter was immersed in 3.5 ml of DNase buffer containing 50 μg of carrier tRNA and 100 μg of DNase and incubated 45 to 90 min at 30°C. Forty to 90 per cent of the radioactivity was released into the supernatant by this digestion. The RNA that remained bound to the filter could not be released by a second DNase treatment. After removing the filter, DNase was inactivated by treatment with 4 μl of DEPC for 10 min at 37°C, and the solution was filtered through a nitrocellulose filter which had been previously rinsed with H2O. The filter was rinsed with 1/4 vol 1 M sodium acetate, pH 5.4. The RNA was precipitated from the filtrate with ethanol. Filtration through nitrocellulose was found to be necessary in order to remove some material (possibly denatured protein) which otherwise interfered with resuspension of the RNA after the ethanol precipitation.

Our initial hybridization experiments followed the procedure of Bövre and Szybalski [42] and Lozorzen. This procedure differed from the procedure described above as follows. For hybridization, RNA was resuspended in 1.5 ml of RNA diluent (0.5 x SSC, 3% phenol) and mixed with 0.15 ml of DNA strands in screw cap vials and incubated at 67-68°C for 4 to 5 hours. To elute the RNA, the filters were heated in 4 ml of H2O at 90°C for 1 min. DNase digestion was then carried out on the released RNA, using the conditions described above for the DNase treatment of the RNA-DNA hybrids, followed by heating at 100°C for 10 min to inactivate the DNase.

**Analytical Hybridization**

Analytical hybridization was carried out by the scale of the preparative hybridization. After RNAase treatment and washing, the filters were counted in 20 ml scintillation vials, containing 8 ml of 2 x SSC by Cerenkov radiation.

**RESULTS**

**Rationale for Use of Read-through, in Vitro Transcription—**

Read-through transcription (transcription initiated upstream at the promoter of another operon) was utilized because it has the following advantages over transcription initiated at the promoter under investigation.

1. It is uniform over the entire target DNA region, whereas initiation controlled by the signals under study is likely to take place within the target region, leaving part or all of the target sequence untranscribed (18, 20).

2. It is independent of the physiological activity of the signals under study. Therefore, the nucleotide changes produced by point mutations affecting the signals' function can be determined merely by introducing them into the DNA template and sequencing the purified transcript (43).

3. It can take place in both directions over the target DNA region, depending on the choice of template. The availability of RNA complementary to both strands greatly facilitates the sequence analysis (43). For read-through transcription to be useful, RNA polymerase must not recognize any transcription termination signals within the target region in either direction.

**In vitro Generation of the RNA has the following advantages over in vivo transcription.**

1. RNA synthesized in vitro can be radioactively labeled at high specific activities more easily than can in vivo RNA, so that only very small physical quantities of RNA (10^-19 mol of phosphate) need be isolated.

2. In vitro RNA can be labeled with only one α-(32P)-ribonucleoside triphosphate at a time which allows nearest neighbor analysis. This facilitates the sequence determination.

3. Initiation at a promoter within the target region, which
would lead to non-uniform transcription of the target region, can be avoided for the lac promoter region in in vitro experiments by leaving the catabolite gene activator protein and cyclic adenosine 3',5'-monophosphate out of the transcription reaction (44, 45).

Choice of Templates—The DNAs of five lac transducing phages were tested for their ability to provide read-through transcription over the lac operon in vitro with E. coli RNA polymerase. Table II shows the results of analytical hybridization of total transcripts of Xh80dlac, Xplac5, and $\phi$0placI DNA. In each case the amount of lac-specific RNA transcribed from either strand was measured by hybridization with a lac-transducing phage DNA of different genetic makeup. RNA hybridizing to the parent phage DNA was subtracted as background.

It can be seen in Table II that Xh80dlac DNA promoted a reasonable amount of LAC (2 to 7%) but no significant CAL transcription. Xplac5 DNA gave rise to a transcript that is 18.5% lac-specific in the LAC direction. This transcription is presumably initiated chiefly at pL (see Fig. 2a), $\phi$0placI DNA promoted transcription backward over the lac operon (see Fig. 2b) to give CAL RNA. In addition to the DNA templates mentioned in Table II, two other phage DNAs were found to be useful for generating lac-specific RNA: $\lambda$ptrp-lac phage DNA for LAC RNA, and $\lambda$ptrp/lac phage DNA for CAL RNA.

Sequential Hybridization Procedure—Two preparative hybridization steps were employed to purify RNA complementary to the lac p-o region. The process is diagrammed schematically in Fig. 2. In the first step, the total transcript was hybridized to the appropriate strand of $\lambda$ptrp/lac X8555 DNA (L strand for CAL RNA and the R strand for LAC RNA). RNA that could not be hybridized was removed by digestion of the heteroduplexes with pancreatic RNase A, which trimmed the lac RNA down to the edge of the X8555 deletion. All phage-coded sequences complementary to the strand not used in this first step were also removed. After inactivation of the RNase by iodoacetate treatment, the RNA was eluted from the hybrid complexes and hybridized in the second step to Aplac5 or $\lambda$ptrp-lac DNA (R strand for CAL RNA and L strand for LAC RNA). RNase treatment then removed the RNA up to the edge of the deletion on the i side of the target sequence.

It is important to note that opposite strands of the transducing phages are employed in the two hybridization steps because the lac DNA is carried in opposite orientations on the hybridization DNAs. This allows complete removal of all phage-coded sequences occurring in the transcript, even when the template and hybridization DNAs are both from the same parent phase (X).

Hybridization conditions—Our initial experiments utilized a procedure similar to that of Bøvre and Szybalski (42), and Lozeron. This procedure has been extensively used for analytical hybridization assays and, by modifying the RNA elution procedure as will be described later, this technique was used successfully to purify RNA 20-fold from undesired sequences in preparation for a sequence analysis (15). However, because the region of the transcript which we wished to purify was less than 0.1% of the total transcript, we found that it was necessary to modify this protocol in order to achieve sufficient purification and recovery. Specifically, we eliminated three high temperature steps in favor of low temperature procedures. These low temperature steps resulted in RNA which: (a) hybridized with greater efficiency and consequently was available in higher yield after multiple preparative hybridization steps, (b) was more specific, and (c) was more intact. The high temperature steps and our modifications of them are the following.

1. DNA-RNA hybridization was initially carried out by an incubation at 67-68°C for 4 to 5 hours in 3% phenol and 0.5 M SSC plus the CsCl contained in the strand preparations (42). We found the use of these conditions resulted in a 10 to 30% loss of hybridizable RNA at each hybridization step relative to hybridizations carried out as described below. Low temperature hybridization is commonly carried out using formamide (46-48) or dimethylsulfoxide (49) to lower the melting temperature of RNA-DNA hybrids. Fig. 3 shows a graph of per cent formamide versus efficiency of liquid-liquid hybridization at 37°C for 20 hours under our chosen salt conditions. It was found convenient to rely almost entirely on CsCl present with the DNA strands to supply the salt necessary during the hybridization. Fig. 3 shows that the optimum formamide concentration is 50%. Our actual operational conditions, as indicated in Fig. 3 and described under "Materials and Methods," are 42% formamide, about 1 M CsCl, 2.5% phenol, and 0.02 M Tris-HCl, pH 7.9.

2. Originally, RNA was eluted from the hybrids by heating...
Diethylpyrocarbonate for 10 min at 37°C. DEPC also destroys DNase. Two-dimensional fingerprints of T1 ribonuclease dinucleotides of RNA prepared using the high temperature elution procedure, for only RNA bound to the nitrocellulose filters by virtue of being hybridized to DNA should be released by RNase treatment after the first hybridization defines the z side of the target RNA and removes phage sequences complementary to the DNA strand not used in this step. RNase treatment after the second hybridization defines the i side of the RNA and removes phage sequences complementary to the strand used in the first step since opposite phase DNA strands are used in the two steps. a, isolation of LAC RNA; b, isolation of CAL RNA; --, RNA; ---, DNA.

Fig. 3. Determination of optimum formamide concentration for RNA-DNA hybridization. RNA which was previously transcribed from λptrp/IacX8554 DNA and preparatively hybridized to λptrp/lacX8555 L strands by the low temperature procedure (see "Materials and Methods") was rehybridized to separated strand of λptrp/lacX8555 DNA in this experiment. The opposite (R) strands were utilized as negative controls. Each hybridization vial contained 15 μl of DNA strands in 6 M CsCl, 15 μl of RNA in H2O, 0.02 M Tris-HCl, pH 7.9, and formamide and water to a total volume of 100 μl with the indicated varying formamide concentration. Incubation was for 20 hours at 37°C, and the results are expressed as the percentage of the input radioactivity which remained bound to the nitrocellulose filters after the filtering and RNase treatment described under "Materials and Methods." for 4 min at 90°C in H2O. This was replaced by a 45-min incubation with DNase at 30°C (see "Materials and Methods"). Theoretically, DNase treatment increases the specificity of the procedure, for only RNA bound to the nitrocellulose filters by virtue of being hybridized to DNA should be released by DNase. Two-dimensional fingerprints of T1 ribonuclease digests of RNA prepared using the high temperature elution technique had a higher level of background contamination than RNA eluted by DNase (data not shown).

3. DNase inactivation by heat denaturation at 100°C for 10 min was replaced by mild, short treatment with 4 μl/ml of diethylpyrocarbonate for 10 min at 37°C. DEPC also destroys any RNase present in the preparation. Parallel hybridization experiments on the two RNA preparations showed that the heated RNA rehybridized with 53% to 76% of the efficiency of the DEPC-treated RNA. Although DEPC has been found by others to derivatize nucleosides under harsher conditions (50) and to destroy the infectivity of tobacco mosaic virus RNA under milder conditions (51), we have never noticed damage to RNA during sequence analysis, even after the three treatments we routinely used; there is no noticeable unusual chromatographic mobility, low yield, or resistance to nucleases on the part of any oligonucleotides in the sequence being analyzed. DNase inactivation by heat can also be avoided by elimination of the DNase treatment and boiling entirely. Blattner and Dahlberg (20) found that no interfering DNA is released from filters when RNA is eluted by heating at 90°C for 4 min, and that if the preparation was not boiled no objectionable RNA degradation was seen. Generally, the removal of radioactivity was better than 95%, although 90°C elution does not take advantage of the probable increased specificity of the DNase elution described above.

Analysis of Final Product—Table III lists the results of analytical hybridization of the final lac promoter-operator RNA isolated by both of the preparative hybridization procedures described in this work. The regions of the lac operon carried by the transducing phage DNAs used in Table III are delineated in Fig. 1. The desired RNA should hybridize to some extent to the appropriate strand of the DNA of φ0placI, λptrp/IacX8555, λrplac5, λptrp-lac F36a, and λptrp-lac W227, because these phages carry all or part of the lac genetic control region. The RNA should not hybridize to λptrp-lac 189 DNA, which contains only the lac z gene, nor to λ or φ080 DNA. The level of hybridization to λptrp-lac 189 DNA (25%) and the over-all low efficiency of hybridization (31%) exhibited by CAL RNA prepared by the high temperature procedure (Column 2) compare unfavorably with CAL RNA prepared by the low temperature procedure (Column 3). Both the CAL and LAC promoter-operator RNA prepared by the low temperature procedure can be seen to be at least 90% pure and contain negligible contaminating λ, φ080, or lac z gene sequences. Possible origins of the remaining contamination will be treated in the discussion. Deletion of a portion of the promoter in the hybridization probe decreases the amount of RNase-resistant RNA hybridized as expected (see the results of the CAL and
The data are listed as the percent hybridizable radioactive RNA hybridizing to the indicated left or right strands of the phage DNAs listed in column 1. The headings of the other columns list the template DNA, the α-35P-nucleoside triphosphate used, the first DNA used for preparative hybridization, and the second DNA used for preparative hybridization. Also indicated is the procedure (high temperature or low temperature) followed during the preparation and analysis (see "Materials and Methods"). The next to last line indicates the input cpm for each datum in the column as measured by Cerenkov radiation. The last line indicates the maximum efficiency of hybridization observed for each RNA. For the RNA analyzed in Column 2 this is the amount of RNA hybridized to λptrp lac W227 1 + r strands. In theory, an equivalent amount should have hybridized to φ80plac 1 + r strands and λptrp/ lac X8555 1 + r strands but, for unknown reasons, it did not. For the RNA analyzed in Columns 3 and 4, the maximum efficiency of hybridization was determined by the amount hybridized to λplac5 1 + r strands.

| Test DNA | φ80plac1-L | φ80plac1-R | λplac5-L | λplac5-R | φ80vir-L | φ80vir-R | λc1,857,57-L | λc1,857,57-R | λptrp-lac L89-L | λptrp-lac L89-R | λptrp-lac W227-L | λptrp-lac W227-R | λptrp-lac F36a S20-R | λptrp-lac F36a-L | λptrp-lac F36a-R | λptrp-lac X8555-L | λptrp-lac X8555-R |
|----------|-------------|-------------|-----------|-----------|-----------|-----------|---------------|---------------|-----------------|-----------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|
|          | λptrp/ lac | λptrp/ lac | λptrp/ lac | λptrp/ lac | λptrp/ lac | λptrp/ lac | λptrp/ lac | λptrp/ lac | λptrp/ lac | λptrp/ lac | λptrp/ lac | λptrp/ lac | λptrp/ lac | λptrp/ lac | λptrp/ lac | λptrp/ lac | λptrp/ lac |
|          | CTP        | UTP         | CTP        | UTP         | CTP        | UTP         | CTP        | UTP         | CTP        | UTP         | CTP        | UTP         | CTP        | UTP         | CTP        | UTP         | CTP         |
|          | high       | low         | high       | low         | high       | low         | high       | low         | high       | low         | high       | low         | high       | low         | high       | low         | high       |
|          | temperature| temperature| temperature| temperature| temperature| temperature| temperature| temperature| temperature| temperature| temperature| temperature| temperature| temperature| temperature| temperature| temperature| temperature|
| Input cpm| 430         | 950         | 4400       |            |            |            |            |            |            |            |            |            |            |            |            |            |            |
| Efficiency of hybridization | 31% | 74% | 62% |            |            |            |            |            |            |            |            |            |            |            |            |            |            |

LAC promoter-operator RNA annealed to λptrp lac F36a DNA.

Fig. 4a shows the two-dimensional separation (fingerprint) of T1 ribonuclease digests of CAL p-o RNA prepared by the high temperature technique. Above a significant background of random RNA can be seen a pattern of a unique RNA of the expected complexity (≥150 nucleotides). However, the larger oligonucleotides (cf. spots 19 to 26 in Fig. 4b) are invisible or at best present in greatly reduced yield. The absence of the large oligonucleotides makes this RNA useless for sequence analysis.

RNase T1 fingerprints of RNA prepared by the low temperature procedures are shown in Fig. 4, b-d. The random background is greatly decreased, and, most important, the yield of large oligonucleotides has increased to a molar ratio consistent with the yield of small oligonucleotides. Thus, RNA prepared by the low temperature procedure is suitable for sequence analysis.

The size of LAC p-o RNA purified by the low temperature procedure was analyzed on 10% polyacrylamide gels. As shown in Fig. 5, there are the three major sizes of RNA in the sample. The largest is about 140 nucleotides long, and the two smaller ones are about half that size. Fingertips of these three bands of RNA show that the two smaller ones are derived from the larger one by nucleotide cleavage. The cleavages most likely occurred during either the DNase or RNase treatments used in the purification procedure since the DNase may not have been entirely free of RNase, or the RNA in the RNA-DNA hybrids may not be completely resistant to the pancreatic RNase used to digest unhybridized RNA, or both possibilities obtain.

Yield of lac p-o RNA—Generally 0.2 to 0.5% of the RNA transcribed from a φ80plac1 template hybridized to L strands of λptrp/lac X8555 DNA at the first hybridization (see Table IV). Of the RNA that was released from λptrp/lac X8555 RNA-DNA hybrids by DNase and used for the second hybridization with λptrp-lac R strands, 5 to 25% hybridized to λptrp/lac X8630 or λplac5 DNA. This indicates that 0.023 to 0.067% of the total transcript is homologous to lac p-o. However, the actual yield of CAL p-o RNA was only 13 to 78% of that expected. Most of the apparent loss of RNA occurred at the DNase elution step, since as much as 50% of the RNA on the filter was not released. Some of this unreleased RNA may be nonspecifically bound to the filter, or this figure may simply reflect difficulty in digesting DNA in bound form. The yield of LAC p-o RNA from λplac5 DNA was higher than the yield of CAL p-o RNA from a φ80plac1 template, corresponding to increased transcription of the lac region from λplac5 DNA. The final RNA was purified 1500- to 6700-fold.

Partial Deletions—By using DNA strands from a λptrp-lac phage carrying a genetic deletion of the lac p or o region for the second or third RNA-DNA hybridization step, we were able to specify the end points of the purified RNA product. The effect of these deletions can be seen by comparing the RNase T1 fingerprints shown in Fig. 4, b-d. For example, the smaller RNA whose end points are defined by deletions F36a and S20 (Fig. 4d) has a fingerprint pattern similar to the larger piece of RNA in Fig. 4b, but it lacks several oligonucleotides found in the larger RNA. These results confirm the conclusion from the analytical hybridization experiments described in Table III.
Fig. 4 (top). Fingerprints of purified lac p-o RNA: suitability for sequence analysis and the effect of partial deletions. CAL p-o RNA was prepared from an [α-32P]CTP-labeled transcript of φ80pLacI DNA using either the high temperature procedure (Fig. 4a) or the low temperature procedure (Fig. 4, b–d) as described under "Materials and Methods." Two-dimensional separations of complete T1 ribonuclease digests of the RNA were prepared as described by Sanger et al. (52). The deletions applied during the hybridization procedure (see Fig. 2b) are indicated beneath each figure. Unfortunately, the pictured deletions do not allow the unambiguous localization of the missing oligonucleotides. These localizations can be made with more appropriate comparisons. Figs. 2b and 2c are identical with ones shown in Dickson et al. (43) and are reproduced with permission of the American Association for the Advancement of Science.

Point Mutations—Point mutations introduced into the template φ80pLacI DNA do not noticeably affect the ability of the transcript to hybridize throughout the procedure (see Table IV). The procedure can, therefore, be applied without modification to the study of point mutations located in any of the genetic control signals under study.

DISCUSSION

We have depended heavily on previous work involving preparative in vitro RNA preparation and on work in lac genetics to develop a technique which permitted the generation of RNA suitable for use in determining the nucleotide sequence of the lac genetic control signals. Our sequential hybridization procedure is highly reproducible and allows the isolation of RNA homologous to either strand of the DNA region extending from near the end of the i gene to the beginning of the z gene. Existing multi-step RNA-DNA hybridization procedures have been used successfully for purifying RNA transcripts up to

Table IV

<table>
<thead>
<tr>
<th>Prep No.</th>
<th>RNA hybridizing at Step 1 to Xplac5-R, X6830-L strands</th>
<th>RNA hybridizing at Step 2 to Yptrp-lac Xplac5-R strand</th>
<th>Experimental yield of CAL p-o RNA from Col 2 × Col 3 transcript</th>
<th>Actual yield of CAL p-o RNA for sequencing Col 4</th>
<th>Recovery Col 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>23. (+)</td>
<td>0.54</td>
<td>12.4</td>
<td>0.067</td>
<td>0.013</td>
<td>19</td>
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<tr>
<td>42. (+)</td>
<td>0.21</td>
<td>6.9</td>
<td>0.015</td>
<td></td>
<td></td>
</tr>
<tr>
<td>44. (p L305)</td>
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<td>12.4</td>
<td>0.036</td>
<td>0.0028</td>
<td>78</td>
</tr>
<tr>
<td>46. (pr19)</td>
<td>0.33</td>
<td>19.5</td>
<td>0.064</td>
<td>0.0035</td>
<td>55</td>
</tr>
<tr>
<td>50. (+)</td>
<td>0.24</td>
<td>15</td>
<td>0.036</td>
<td>0.0126</td>
<td>17</td>
</tr>
<tr>
<td>52. (p L305)</td>
<td>0.50</td>
<td>4.7</td>
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<td>0.0031</td>
<td>13</td>
</tr>
<tr>
<td>116. (p D640)</td>
<td>0.27</td>
<td>25</td>
<td>0.066</td>
<td>0.0011</td>
<td>17</td>
</tr>
</tbody>
</table>
20-fold relative to unwanted RNAs for sequence analysis (15); however, in order to accomplish the necessary 1500- to 6700-fold purification in this case, these procedures were modified by eliminating all high temperature steps. The final RNA prepared by our modifications is at least 90% pure and 50% intact.

In vitro transcription from whole transducing phage DNA was used to generate radioactively labeled RNA. Had we used as templates smaller DNA molecules, such as those produced by restriction endonucleases (58), the subsequent purification procedures need not have been so powerful.

Our purification technique in its present form requires the construction of two sets of specialized transducing phages which carry deletions defining the target region on each boundary. In addition, in order for the technique to eliminate phage-homologous sequences, it is necessary to have the target DNA carried in opposite orientations on two sets of phages. In the experiments described here, we have combined these requirements by utilizing deletion-carrying phages that also carry the lac DNA in opposite orientations. However, since the isolated RNA can survive an additional hybridization step, the oppositely oriented operon could have been carried on a third transducing phage having no defining deletions.

Since the technique utilizes read-through transcription of the target DNA region, our method allows the sequence analysis of regions of the DNA which are not normally transcribed.

Previous structural studies of genetic control signals in the lac region have relied on the physiological activity of the signals under study, such as binding or repressor (17) or transcription initiated by the lac promoter (18). A great advantage of our method is that it is indifferent to point mutations introduced into the in vitro template. Thus the nucleotide change produced by a point mutation can be determined by using a mutant DNA template followed by our standard isolation of the p-o region. By applying standard sequencing techniques we have determined the nucleotide changes caused by several lac promoter mutants using this technique (43).

This method did not depend on any previous partial knowledge of the sequences in or near the target region, as would be required for DNA sequencing utilizing a synthetic oligonucleotide primer (58). Cleavage sites recognized by restriction endonucleases are neither required nor utilized by the technique in its present form although such cleavages could be used to supplant genetic deletions during hybridization in a modification of the technique.

The successful elimination of all phage-homologous sequences puts a limit on the similarity and symmetry of sequences transcribed from our transducing phage DNA templates in vitro. The smallest piece of RNA that survives the procedure is 35 to 40 nucleotides long. Therefore, the lac promoter is not identical to any phage sequences that are 35 nucleotides long. ADNA sequences on the template having regions of perfect or near perfect 2-fold symmetry involving at least 35 base pairs would also give rise to RNA that would survive our purification procedure. Apparently such sequences do not exist in the region of the templates that are transcribed under our conditions.

Ten per cent of purified lac p-o RNA hybridizes to the wrong strand of DNA (see Table III). This could have three explanations: (a) Extensive partial symmetry in the lac operator region (17, 43) might allow inefficient hybridization to the opposite DNA strands. (b) The recently discovered second repressor binding site in the z gene (35) may contain DNA sequences that can hybridize to some extent with lac operator RNA. The lack of hybridization to the homologous strand of a ptrp-lac 189 DNA (a deletion which does not remove the second binding site) indicates that this sequence would have to be an inversion if it can hybridize the RNA at all. (c) Cross-contamination in the separated strand preparations. Our data are insufficient to allow us to distinguish among these possibilities.

We have successfully used aplac5 and a ptrp-lac W2 DNA as templates for the synthesis of LAC RNA. This indicates that RNA polymerase is capable of transcribing across the end of the i gene and into the p-o region. Therefore, if there is a transcription stop signal at the end of i, it is not recognized efficiently in our in vitro system. Recent in vivo experiments also suggest that there may be no mRNA stop signal at this location (21).

The complete nucleotide sequence of the lac promoter-operator region has been determined using RNA prepared by the technique described here (43).

REFERENCES

34. Wiberg, J. S. (1958) Arch. Biochem. Biophys. 73, 337-358