THE EFFECT OF CHLORAMPHENICOL ON SYNTHESIS OF ΦX 174-SPECIFIC PROTEINS AND DETECTION OF THE CISTRON A PROTEIN

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SUMMARY

Synthesis of ΦX 174-specific proteins in *Escherichia coli* H 502 was examined on sodium dodecyl sulphate–acrylamide gels by coelectrophoresis of proteins from [³H]leucine-labelled infected cells and [¹⁴C]leucine-labelled reference cells, which had been infected with ultraviolet-light irradiated phage. Addition of 35 μg/ml of chloramphenicol during infection reduced the synthesis of ΦX-specific proteins of cistrons A, F, H and D to different degrees. The greatest reduction is found in synthesis of the cistron F product (coat protein), while synthesis of cistron D protein (single-strand DNA synthesis) is relatively resistant to the drug. Mutants in cistron A, which are unable to replicate RF, show a great reduction in the synthesis of all ΦX-specific proteins upon infection in the presence of chloramphenicol which made the identification of the cistron A protein unfeasible under these conditions.

In the absence of chloramphenicol the cistron A product was identified as a protein with a molecular weight of 55 000. The cistron A protein is always strongly associated with the cell membrane.

INTRODUCTION

It is well known that for the initiation of DNA replication protein synthesis is required. The identity of the proteins involved and their function in DNA replication is only partially understood[1]. The synthesis of such proteins involved in the initiation of DNA replication in *Escherichia coli* and the phages ΦX 174, S 13 and λ is often called chloramphenicol resistant[2-5], since this process is relatively unaffected by concentrations of the antibiotic sufficient to block overall protein synthesis almost completely.

Levine and Sinsheimer[6-8] identified a ΦX-specific chloramphenicol-resistant protein of a molecular weight of approx. 14 000 in ΦX-infected cells and these authors presented evidence that this protein corresponds to cistron A (according to the nomenclature of Sinsheimer and Hayashi[9]) of the ΦX genome. A functional cistron A product is required for the initiation and continuation of ΦX RF DNA replication[10]. However, other investigators assigned molecular weights of approx. 25 000 (ref. 11) and 60 000 (ref. 12) to the cistron A product.

Abbreviation: RF, double-stranded replicative form DNA.

Because of these contradicting results we have reinvestigated the problem aiming at the detection of cistron A protein in the presence of chloramphenicol as well as in the normal infection process, thus avoiding the artificial conditions of ultra violet-light irradiated *hcr*⁻ hosts as used by others[11,12]. ϕX-specific proteins were detected in lysates of infected cells using a double-label technique and electrophoresis of the proteins in sodium dodecyl sulphate–polyacrylamide gels.

Among other ϕX-specific proteins (corresponding to cistrons F, H, G, D and C) a protein of a molecular weight of 55 000 was detected which is specifically absent in cells infected with amber mutants in cistron A of the ϕX phage. The location of the cistron A product in the cell and the effect of chloramphenicol on the synthesis of ϕX-specific proteins was investigated.

MATERIALS AND METHODS

Bacterial strains and ϕX 174 mutants

The ϕX-sensitive *E. coli* strains were obtained from Dr R. L. Sinsheimer. *E. coli* H 502 is a *uvrA*⁻, *thyA*⁻, end I⁻ strain. *E. coli* HF 4712 is a permissive host for amber mutations. ϕX 174 wild type and the amber mutants am 42 (a cistron D mutant), am 33 and am 18 (both cistron A mutants) were obtained from Dr R. L. Sinsheimer. Amber mutant phage stocks were always less than 1 ‰ with respect to wild-type revertants.

Media

Growth medium contains per 1: 0.5 g NaCl, 1.1 g NH₄Cl, 0.5 g MgCl₂ · 6H₂O, 1.0 g KH₂PO₄, 12 g Tris, 0.8 g sodium pyruvate and 0.02 g Na₂SO₄. The pH was adjusted to 7.4. After autoclaving the following sterile solutions were added: 1 ml of 1 M CaCl₂, 1 ml of a solution which contains 0.1 mg/ml FeCl₃ · 6H₂O, 20 ml of 10 % glucose and 10 ml of a solution which contains 20 mg/ml casamino acids (Difco).

In experiments in which radioactive leucine was used as a label for protein synthesis casamino acids were replaced by an amino acid mixture in which leucine was missing.

In experiments in which thymine-requiring strains were used, thymine was added to a final concentration of 5 μg/ml.

Cells were washed in a buffer with the same composition as growth medium, except for the carbon- and nitrogen-containing compounds.

Isotopically labelled compounds

L-[4,5-³H]Leucine and L-[U-¹⁴C]leucine were obtained from the Radiochemical Centre, Amersham, England; [6-³H]thymidine from CEN-SCK, Mol. Belgium.

Infection and labelling of cells

Cells were grown to a cell density of 2 · 10⁸/ml, spun down and washed once. For infection in the presence of chloramphenicol the cells were resuspended in 1/10 of the original volume of buffer *plus* 35 μg/ml chloramphenicol.

Half of the cells were infected with ϕX 174 at the desired multiplicity of infection. The other half received ϕX 174 which had been irradiated with ultraviolet light

at a dose of 1600 ergs/mm², which is high enough to assure that the synthesis of ϕX-specific proteins is completely blocked.

Using ultraviolet-light irradiated phage-infected cells instead of uninfected cells as a reference, enhanced the reproducibility of the gel patterns and facilitated the detection of ϕX-specific proteins. After 5 min at 37 °C without aeration to adsorb the phages to the cells, prewarmed growth medium *plus* 35 μg/ml chloramphenicol was added to restore the original cell density and aeration was resumed. In all chloramphenicol experiments this moment was taken as the onset of the process of infection. The ϕX-infected culture was labelled in all experiments with [³H]leucine (20 μCi/ml, spec. act. 2.5 Ci/mmole), the reference culture received [¹⁴C]leucine (2.5 μCi/ml, spec. act. 311 Ci/mole). To stop incorporation, the cells were poured in an equal volume of ice-cold buffer, which contained 0.1 M KCN and 1 mg/ml of cold leucine.

Preparation of the labelled cells for polyacrylamide gel electrophoresis

The cells of the infected and reference culture were combined and centrifuged in the cold. After lysis of the cells according to the osmotic shock procedure of Knippers and Sinsheimer[13] the lysate was centrifuged at 7000 × g for 1 h. The supernatant was made 0.02 M in MgCl₂ and 0.01 M in mercaptoethanol. The viscous pellet was resuspended in a small volume of a buffer containing 0.01 M Tris, 0.003 M MgCl₂ and 0.01 M mercaptoethanol, pH 7.4. Both pellet and supernatant were incubated with deoxyribonuclease (40 μg/ml) during 10 min at 37 °C. Sodium dodecyl sulphate and urea were added to final concentrations of 1 % and 0.5 M, respectively, followed by incubation for 1 h at 37 °C. This incubation gave clear solutions of both pellet and supernatant. Finally both solutions were dialyzed against several hundred volumes of a buffer containing 0.01 M sodium phosphate, 0.1 % dodecyl sulphate and 0.01 M mercaptoethanol, pH 7.4.

Polyacrylamide gel electrophoresis and counting of radioactivity

Gel electrophoresis was performed in glass tubes. The gels were 9 cm in length and 6 mm in diameter, and consisted of 10 % acrylamide (K and K Laboratories), 0.27 % ethylenediacrylate (w/v) (K and K Laboratories), 0.05 % N, N, N', N'-tetra-methylethylenediamine (v/v) (TEMED; Eastman Organic Chemicals), 0.1 % sodium dodecyl sulphate and 0.075 % ammonium persulfate in 0.1 M sodium phosphate buffer, pH 7.2. The sample (routinely 100 μl) was applied in 20 % sucrose and electrophorized during 1 h at 5 mA per tube, followed by 10 mA per tube for 5–7 h.

Electrophoresis was stopped when the tracking dye, bromophenol blue, had run approx. 7 cm. The gels were removed from the tubes, frozen on solid CO_2, and sliced in a cooled microtome in slices of 1-mm thickness. The slices were solubilized in 0.5 ml 0.9 M NH₄OH in a scintillation vial by shaking for 12 h at 37 °C. For radioactive counting, 14.5 ml of a 6 : 23 (v/v) mixture of Triton X-100 and a toluene solution of PPO and POPOP was added. Radioactivity was measured in either a Mark I or Mark II scintillation counter of Nuclear Chicago, with proper settings for simultaneous counting of ³H and ¹⁴C.

RESULTS

(a) Synthesis of ϕX-specific proteins in the presence of chloramphenicol

In the presence of 35 μg/ml chloramphenicol RF replication proceeds at a normal rate[14], while overall protein synthesis is inhibited for at least 90 %. As synthesis of cistron A protein is needed for RF replication it can be concluded that cistron A protein is either needed in much smaller amounts than those synthesized in normal infection or that its synthesis is relatively unaffected by the presence of chloramphenicol.

Fig. 1 shows the effect of chloramphenicol (35 μg/ml) on RF replication using an amber mutant of ϕX 174 in cistron D(am 42). This mutant performs a normal RF replication with a shut off at approx. 10 min after infection, but no single-strand progeny DNA synthesis occurs[15]. It can be seen that RF replication in the presence of chloramphenicol starts later than RF replication in the absence of the drug and is not followed by a shutt off. The rate of RF synthesis is unaffected by chloramphenicol.

Proteins synthesized in the presence of chloramphenicol during a 60-min labelling period from [14C]leucine-labelled uninfected cells and [3H]leucine-labelled infected
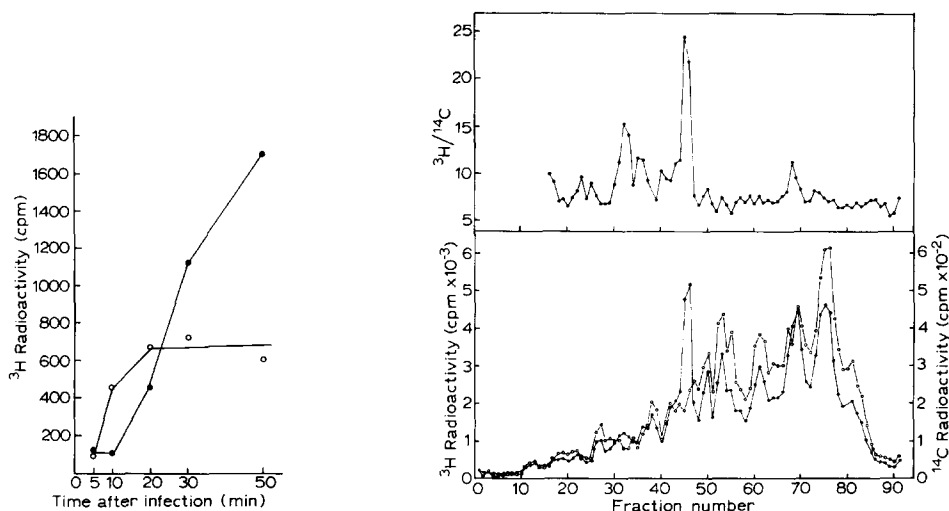

Fig. 1. Effect of 35 μg/ml of chloramphenicol on RF replication. *E. coli* H 502 was grown to a cell density of 2 · 10^8/ml and treated with 25 μg/ml of mitomycin C to suppress host DNA synthesis[26]. After one wash the cells were resuspended in a small volume of wash buffer and divided into two portions, each of which was infected with ΦX am 42 (multiplicity of infection of 10). One portion received in addition 35 μg/ml of chloramphenicol. After 10 min at 20 °C without aeration, growth medium containing 4 μCi/ml of [3H]thymidine was added. The chloramphenicol-containing portion was brought to a final concentration of 35 μg/ml and aeration was resumed at 37 °C. Incorporation of label into DNA was measured by determining the acid-insoluble alkali-resistant counts in samples withdrawn as indicated in the figure. ●–●,35 μg/ml of chloramphenicol; ○–○, no chloramphenicol.

Fig. 2. Electrophoresis pattern of labelled proteins from the pellet. Protein from the pellet, obtained by centrifugation of a lysate of *E. coli* H. 502 cells infected with ΦX 174 (multiplicity of infection of 5) and labelled during 60 min in the presence of 35 μg/ml of chloramphenicol, were extracted and subjected to electrophoresis as described in the text. Top, ratio of 3H to 14C counts in each gel slice: Bottom, number of 3H counts (●, infected cells) and 14C counts (○, reference cells) in each gel slice. The top of the gel is at the left.

cells were extracted by sodium dodecyl sulphate–urea treatment of the pellet and supernatant fraction of the combined cell lysate as described in Materials and Methods.

In Fig. 2 a typical electrophoresis pattern on sodium dodecyl sulphate–polyacrylamide gels of the pellet proteins is shown. Several fractions show a surplus of ^{3}H activity, as revealed by a deviation in ^{3}H/^{14}C ratio. In order to obtain information about the amount of possible phage specific proteins in these fractions the ^{14}C counts present in each fraction were multiplied by the ratio of ^{3}H/^{14}C in the gel regions in which this ratio was minimal. The resulting number represents the number of ^{3}H counts in the fraction as expected when only host proteins had been synthesized in the same amounts as in the reference culture. By subtracting these counts from the number of ^{3}H counts found in the fraction the surplus of proteins synthesized in the infected cell is found.

In Figs 3A and 3B the pattern thus obtained of proteins synthesized in the infected cells during a 60-min labelling period in the presence of chloramphenicol is shown for supernatant and pellet, respectively. These proteins are defined as ϕX specific, *i. e.* they are either coded for by the ϕX genome or they are host proteins the synthesis of which is stimulated by ϕX infection.
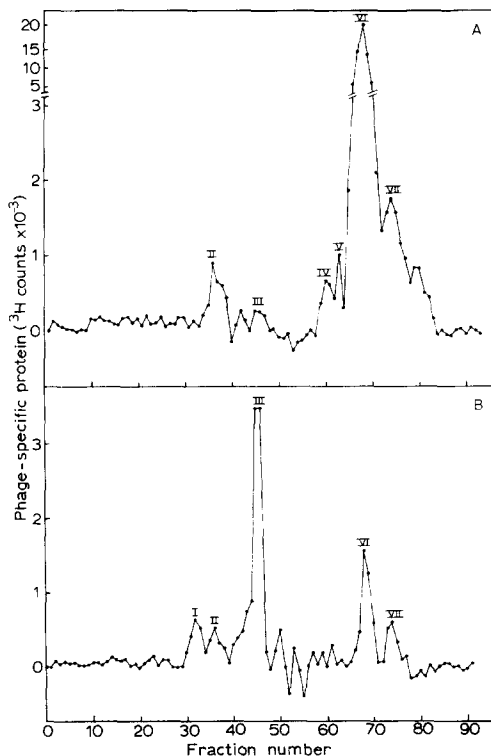

Fig. 3. Phage-specific proteins synthesized in the presence of chloramphenicol. After polyacrylamide gel electrophoresis of a mixture of proteins synthesized in the presence of 35 μg/ml of chloramphenicol during a 60-min labelling period from ΦX-infected cells (^{3}H label) and reference cells (^{14}C label), ΦX-specific ^{3}H counts in each gel slice were obtained as described in the text. (A) Phage-specific proteins from the supernatant fraction obtained after centrifugation of the cell lysate. (B) Phage-specific proteins from the pellet fraction.

In order to determine to which cistrons of the ϕX genome these proteins (Peaks I to VII in Fig. 3) correspond their molecular weight was determined according to the procedure of Shapiro et al.[16] and compared to the known molecular weights of the protein products of the ϕX cistrons (Table I).

TABLE I

MOLECULAR WEIGHT OF THE PEAKS I TO VII IN FIG. 3, AND THE CISTRONS OF THE $\varPhi$X GENOME TO WHICH THESE MOLECULAR WEIGHTS CORRESPOND ACCORDING TO THE LITERATURE CITED

The molecular weights were obtained by comparing the migration of the labelled peaks in the gels with those of proteins with known molecular weight[16]. The marker proteins used were: bovine serum albumin (mol. wt 65 500); pepsin (mol. wt. 35 000); lysozyme (mol. wt 14 300) and ribonuclease T_1 (mol. wt 11 085). The determination of the molecular weights was done in six experiments. In parentheses the max. and min. mol. wt are indicated.

Peak No.	Mol. wt	Probable cistron
I	55 000 (54 000–57 000)	A[12,18]
II	50 000 (48 000–51 000)	F[8,12,19,11]
III	40 000 (39 000–41 500)	H[8,12,19]
IV	23 000 (22 000–23 500)	?
V	19 000 (18 000–20 500)	G[12]
VI	14 000 (13 000–14 500)	D[8,12,19]
VII	9 000 (8 500–10 000)	C[20]

Additional information about the nature of the ϕX-specific proteins was obtained by comparing the gel patterns of the radioactive peaks with the gel pattern of the coat proteins, isolated according to the method of Poljak[17]. The electrophoretic mobility of Peaks II, III and V was the same as that of coat proteins F, H and G, respectively.

Confirmation that the protein Peak VI is the cistron D product stems from the finding that this was the only peak which disappeared upon electrophoresis of proteins of cells which had been infected with a ϕX mutant (am 42) in cistron D in the presence of chloramphenicol.

Upon infection of cells in the presence of chloramphenicol with amber mutants in cistron A (am 18 and am 33) it was found that synthesis of all ϕX-specific proteins is severely depressed. This depression was not found upon infection of E. coli HF 4712, an su$^+$ strain.

(b) Synthesis of ϕX-specific proteins during normal infection

Since it is known that in normal infection (without chloramphenicol) synthesis of most ϕX-specific proteins is not depressed upon infection of su$^-$ cells with amber mutants in cistron A[8,11], the detection of ϕX-specific proteins synthesized during a normal infection was attempted.

Cells were infected with ϕX 174 and labelled with [³H]leucine for 2–6 and 8–12 min after infection, respectively. A control culture was infected with ultraviolet-irradiated phage and labelled during the same time interval with [¹⁴C]leucine.

Proteins were extracted from the pellet and supernatant of the combined lysates as described in Methods. In Figs 4A and 4B the electrophoresis pattern of the ϕX-specific proteins synthesized upon labelling from 8 to 12 min after infection are

shown for pellet and supernatant, respectively. Similar patterns are shown in Figs
4C and 4D for cells infected with am 33. The only peak missing in the am 33 experi-
ment is Peak I in the pellet. The electrophoretic mobility of this peak is the same as
that for Peak I in the chloramphenicol-treated cultures (Fig. 3). Cultures labelled
from 2 to 6 min after infection showed the same ϕX-specific proteins as those labelled
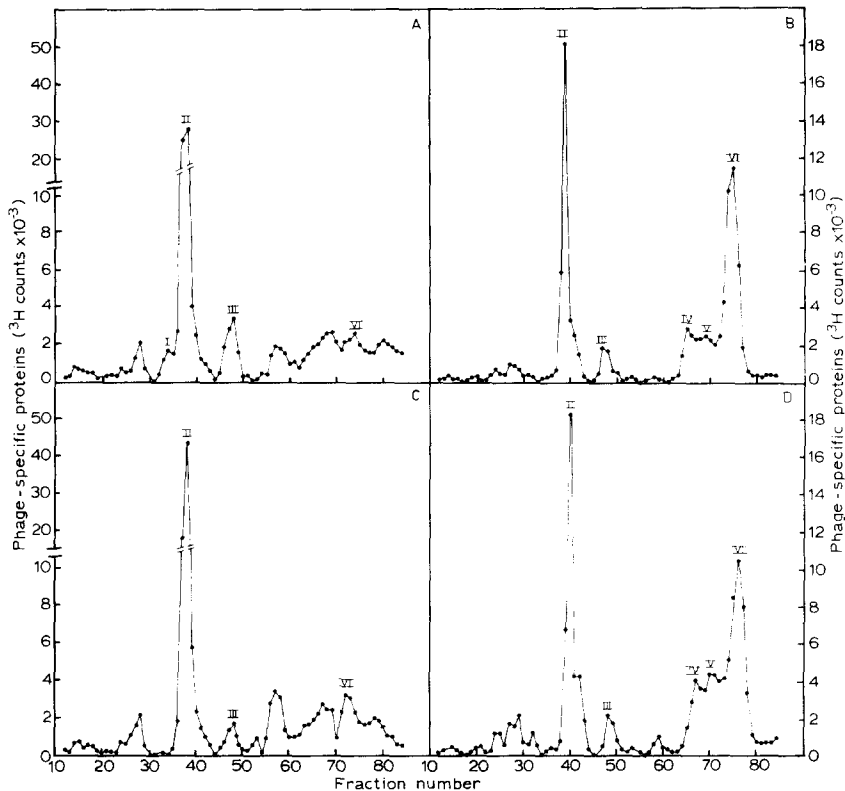from 8 to 12 min after infection, although in smaller amounts.

Fig. 4. Phage-specific proteins synthesized during normal infection. *E. coli* H 502 was infected
with ΦX 174 (multiplicity of infection of 10) in the absence of chloramphenicol and labelled
during 8–12 min after infection with [³H]leucine. The proteins of these cells were co-electropho-
rezed with those of reference cells which had been labelled during the same time period with [¹⁴C]-
leucine. Phage-specific ³H counts in each gel slice were obtained as described in the text. (A)
and (B), phage-specific proteins from wild-type ΦX-infected cells from pellet and supernatant,
respectively; (C) and (D), phage-specific proteins from ΦX am 33-infected cells from pellet and
supernatant, respectively.

The finding that the cistron A product is present exclusively in the pellet
fraction in these experiments suggests a membrane-bound character of this protein.
This is feasible because it is known that replication of ϕX 174 RF DNA is restricted
to the membrane[13]. Attempts to release the labelled proteins from the resuspended
pellet (Fig. 4) using deoxyribonuclease (40 μg/ml), or ribonuclease (20 μg/ml) or a
combination of these enzymes were unsuccessful.

When the pellet was resuspended in 0.01 M Tris, 0.01 M mercaptoethanol, pH 7.4 and sonicated during 4 min (Branson sonifier, full capacity) followed by incubation with sodium deoxycholate (2 %) and the nonionic detergent Brij 58 (0.5 %) during 1 h at 20 °C, about 80 % of the radioactivity in the pellet was released.

Electrophoresis of the labelled proteins remaining in the pellet gave a pattern as shown in Fig. 5A. It can be seen that a relatively large fraction of cistron A protein is still found in the pellet. In confirmation with the conclusions above, the cistron A peak is lacking in the electrophoresis pattern of the sonified pellet from cells infected with ϕX am 33 (Fig. 5B).
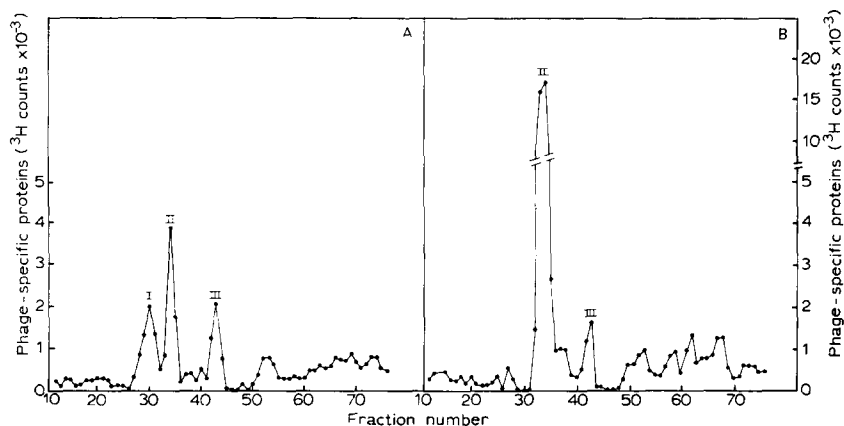

Fig. 5. Phage-specific proteins in the pellet after sonication and treatment with detergents. The pellet, obtained by centrifugation of a lysate of ^{3}H-labelled cells infected with ΦX 174 (multiplicity of infection of 10) and ^{14}C-labelled reference cells, was sonicated and treated with sodium deoxycholate and Brij 58 as described in the text. After centrifugation during 1 h at 8000 × g the resulting pellet was subjected to electrophoresis and phage-specific ^{3}H counts were calculated. (A) Phage-specific proteins from wild-type ΦX-infected cells. (B) Phage-specific proteins from ΦX am 33-infected cells.

(c) Comparison of the amount of ϕX-specific proteins synthesized in the presence and absence of chloramphenicol

From the amount of ^{3}H label present in the ϕX-specific proteins on the gels, the total amount expressed as incorporation of radioactivity in each of these proteins can be determined. This was done for three cultures, each of which was infected with ϕX 174 at a multiplicity of 10.

Two of these cultures were grown in the presence of 35 μg/ml of chloramphenicol and labelled from 0 to 20 min and 0 to 60 min, respectively. The third culture was grown in the absence of chloramphenicol and labelled from 0 to 10 min after infection. During this period the rate of RF replication is maximal and the cistron A protein peak is not obscured by the huge amount of cistron F protein which is synthesized when this period is extended. Moreover, the relative amounts of the ϕX-specific proteins synthesized remain approximately the same throughout infection (unpublished results and refs 8 and 12), so the time during which the cells are labelled is not critical. In Table II the total number of ^{3}H counts incorporated in each of the four well-defined protein Peaks I, II, III and VI (Figs 3 and 4) which correspond to

TABLE II

AMOUNT OF ΦX-SPECIFIC PROTEINS OF CISTRONS A, F, H AND D SYNTHESIZED IN THE PRESENCE OR ABSENCE OF CHLORAMPHENICOL

Data in number of ³H dpm incorporated in 10⁹ infected cells.

Cistron	−Chloramphenicol (0–10)*	+Chloramphenicol (0–20)	+Chloramphenicol (0–60)
A	122 000	13 250 (10.9)**	19 000 (15.6)
F	1000 000	15 000 (1.5)	30 000 (3)
H	275 000	21 000 (7.6)	82 000 (29.8)
D	1300 000	139 000 (10.7)	691 000 (53.2)

* In parentheses the time after infection in min during which the cells were labelled.
** In parentheses the percentage of ³H counts + chloramphenicol with respect to —chloramphenicol.

cistrons A, F, H and D, respectively, is shown for each of the three cultures. The data indicate that the synthesis of cistron F protein is extremely sensitive to the action of the antibiotic and that the small protein of cistron D does escape the action of chloramphenicol more easily than the larger proteins corresponding to cistrons A and H.

DISCUSSION

From the results presented in Sections (a) and (c) it is clear that upon infectioii of *E. coli* H502 in the presence of 35 μg/ml of chloramphenicol the synthesis of ϕX-specific proteins is differentially affected by the action of the drug. Synthesis of the cistron F product, the major coat protein of the phage, is depressed to approx. 1 % of its normal rate. Depression of synthesis of the cistron D protein, which is needed for single-strand progeny DNA synthesis is about 90 % under these conditions. The degree of inhibition for the products of the cistrons A (RF replication) and H(spike protein) is in between that for the products of cistrons F and D. As the switch from RF replication to single-strand DNA synthesis is dependent upon the synthesis of each of the coat proteins of the phage[21], the reason that in low concentrations of chloramphenicol this switch is not observed may relate to the low level of cistron F protein under these conditions rather than to a lack of cistron D product.

Godson[22] showed that in cells which had been irradiated with heavy doses of ultraviolet light prior to infection, synthesis of all ϕX-specific proteins is depressed to approximately the same degree by chloramphenicol. This, however, may be caused by the fact that the protein synthesizing machinery of the host is severely affected by irradiation[23].

The finding that in chloramphenicol the synthesis of all ϕX-specific proteins was reduced upon infection of the cells with ϕX mutants in cistron A, made this system unsuitable for the detection of the cistron A product. As polar effects of mutations in cistron A have not been found, the most likely explanation of this phenomenon is that the amount of ϕX-specitic proteins synthesized under these conditions is related to the number of RF copies present. After infection, amber mutants in cistron A only convert the infecting phage DNA to parental RF, whereas synthesis of progeny RF is blocked.

Cells infected with wild-type ϕX synthesize, in the presence of chloramphenicol, up to several hundred copies of RF[14].

Without chloramphenicol ϕX mutants in cistron A synthesize upon infection in *E. coli* H502 normal amounts of all ϕX-specific proteins, with the exception of one protein, migrating at the 55 000 molecular weight position in the gel, which is in good accordance with the molecular weight of the cistron A protein given by Godson[12] and Iwaya and Denhardt[18].

The fact that it is not possible to release the cistron A product from the pellet by deoxyribonuclease and/or ribonuclease, and that sonication in combination with treatment with mild detergents still leaves a considerable fraction of cistron A radio-activity in pelletable material, suggests a tight binding to membrane components of the cell. Many known features of RF replication are in accordance with such a membrane-bound character of the cistron A product. RF replication is restricted to the membrane, and occurs only at specific membrane sites[24]. Indirect evidence[25] suggests that the cistron A product acts by nicking one of the two strands of double-stranded ϕX DNA, and that this nicking is limited to the replicating RF. Moreover, upon mixed infection of cells with amber mutants in cistron A and wild-type ϕX, the amber mutant is not rescued[4,5], which can easily be explained by assuming that the functional cistron A product of the wild-type phage is not exchangeable from the site where it is synthesized.

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